CHARACTERIZATION OF FOUR ALTERNATIVELY SPLICED CASEIN KINASE I ALPHA ISOFORMS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1999

ACKNOWLEDGMENTS

First of all, I would like to express my gratitude to my mentor, Dr. Gudrun S. Bennett, for providing me with this very precious opportunity to work in a wonderful laboratory, and for her excellent guidance and training during my five-year graduate study. I would also like to extend my gratitude to my committee members – Drs. John P. Aris, Alice C. Harmon, Gerry Shaw, and Stephen P. Sugrue – for their support, advice, and encouragement of my research. I am also grateful to Dr. Brain A. Hollander for his pioneer work in the lab, and Dr. Calvert L. Green, my labmate, for his brilliant collaboration, many valuable discussions, and comments on my research. I am in great debt to all faculty, staff, and student groups at the Department of Anatomy and Cell Biology for the countless help they rendered and the stimulating intellectual environment they provided.

My special thanks are reserved for my mother, Guang-Hua Fu, and my father, Ru-Lian Fu, both professors of physics in China, for giving me the best education ever since I was a child, for their wholehearted support for me, and deeply rooted confidence in me all through these five years.

TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy

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December 1999

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Major Department: Anatomy and Cell Biology

Casein Kinase I (CKI) is a large serine/threonine protein kinase family, consisting of multiple isoforms. Although it is capable of phosphorylating a large number of physiological substrates, very few of them have been characterized in vivo. Also very little is known about the differences in functions and regulation between different isoforms.

Previously it was found that purified neurofilaments (NFs) contained an associated kinase (NFAK) activity that phosphorylates selectively a subset of sites in the tail of chicken NF-M and has properties consistent with CKI. Because CKI consists of as many as seven genes, we investigated the extent to which different CKI isoforms contribute to NFAK activity. During the purification, we determined that NFAK activity copurified with casein kinase activity, and it predominantly occurred at 36-40 kDa, corresponding to the size of CKIα isoforms. Chicken neurons express transcripts encoding four

alternatively spliced variants of CKI alpha (CKIα, CKIαS, CKIαL, and CKIαLS) differing in the presence or absence of two inserts, L and S. We determined that all four isoforms were expressed in chicken brain. However, only CKIα and CKIαS could be detected in purified NFAK, and they together accounted for the entire NFAK activity.

The selective association of CKIα and CKIαS with the neuronal cytoskeleton suggests that the L insert may carry information that renders CKIαL and CKIαLS inaccessible to NFs. Since it contains a putative nuclear localization signal, we then examined if the L insert is capable of directing CKIαL and CKIαLS to the nucleus. All four full-length sequences were cloned into a Green Fluorescent Protein (GFP) vector to generate CKI alpha fusion proteins with a GFP tag, and were transiently transfected into cultured cells. We found that GFP-CKIαL and GFP-CKIαLS proteins were concentrated in the nucleus, whereas GFP-CKIα and GFP-CKIαS proteins were concentrated in the cytoplasm. More interestingly, the two nuclear isoforms completely co-localized with nuclear speckles, and this co-localization still occurred upon inhibition of transcription. In view of the phosphorylation-dependent association of splicing factors with nuclear speckles, our findings suggest that the two nuclear CKI alpha isoforms may be involved in the regulation of RNA splicing and metabolism.

CHAPTER 1
INTRODUCTION TO CASEIN KINASE I

Casein Kinase I (CKI) is a ubiquitously expressed, second messenger-independent, serine / threonine protein kinase that recognizes acidic rather than basic substrates. At this time, it is clear that CKI is not a single kinase, but a family consisting of multiple isoforms. To date, both the function and the regulation of these isoforms still remain poorly understood. On the one hand, although numerous protein substrates for CKI in vitro have been identified, very little is known as to whether or not these potential targets are relevant physiological substrates in vivo, and which specific CKI isoform(s) may be responsible. On the other hand, the CKI activity has been identified in several intracellular locations, but the isoform-specific subcellular compartmentalization is largely uncharacterized. Recently, it has been suggested that the exact localization of several yeast CKI isoforms is absolutely essential for their respective functions and regulation (Vancura et al., 1994).

Among all vertebrate CKI isoforms, CKIα is the best characterized in terms of its localization and potential functions. Still, given that there are as many as four alternatively spliced CKIα isoforms, it is critical to gain a clear understanding of the differences in localization among the four isoforms as a means of further defining their functions and regulation. In the following sections, I am going to review not only what is now known, but also point out what is not known about CKI isoforms, with the attempt to

bring to the spotlight the most fundamental issues in this field that need to be addressed and which are also the focus of the research presented in the subsequent chapters.

Casein Kinase I Isoforms

CKI is a monomer, with an isoelectric point ranging from 9.0-9.6. It requires Mg^{2+} for activity and is strongly activated by monovalent cations. The enzyme prefers ATP over GTP as the phosphoryl donor, with a Km in the range of 7-25 μM. So far, CKI has been identified in all eukaryotes examined from yeast to human, and isolated from cytosolic, membranous and nuclear fractions of mammalian cells. Cytosolic and membranous CKIs are monomers of 30-37 kDa, but nuclear CKIs vary in reported mass from 23-55 kDa (Tuazon and Traugh, 1991).

Since 1991, it has become apparent that CKI is a large protein kinase family, consisting of as many as seven distinct genes in vertebrates, α, β, γ1-3, δ, and ε (Fish et al., 1995; Graves et al., 1993; Rowles et al., 1991; Tapia et al., 1994; Zhai et al., 1992; Zhai et al., 1995). Several vertebrate CKI homologue genes have also been identified in yeast, including HRR25, YCK1, YCK2, YCK3 in S. *cerevisiae* (Hoekstra et al., 1991; Robinson et al., 1992; Wang et al., 1992) and Cki1, Cki2, Cki3, Hhp1, Hhp2 in S. *pombe* (Dhillon and Hoekstra, 1994; Kitamura and Yamashita, 1998; Wang et al., 1994). All CKI isoforms share a similar structure consisting of a conservative 300 amino acid catalytic domain and variably sized N-terminal and C-terminal extensions. The N-terminal domain is usually less than 15 amino acids in length, except CKIγ (43 amino acids) and yeast Yck1 and Yck2 (73 amino acids). On the contrary, the C-terminal

extension ranges in size from 13 to 188 amino acids, and shows little homology among different isoforms (Graves and Roach, 1995).

Vertebrate CKI Isoforms

It was in 1991 that mammalian cDNAs encoding multiple CKI isoforms were first described by Rowles et al. (Rowles et al., 1991). They isolated two full length bovine brain cDNAs encoding CKI isoforms designated CKIα and CKIβ with predicted molecular weight of 37.6 kDa and 38.7 kDa. A partial cDNA was also isolated that encodes a third form designated CKIγ, as well as a 126-bp fragment predicting another isoform termed CKIδ. Since then, as many as seven distinct CKI isoforms (α, β, γ1-3, δ, ε) have been identified. Three genetically distinct isoforms of CKIγ (γ1-3) were originally isolated from rat testis, with predicted molecular masses of 43 kDa, 45 kDa and 49.7 kDa (Zhai et al., 1995). Among all tissues tested, messages for γ1 and γ2 were restricted to testis, whereas messages for γ3 appeared in all except spleen and heart. At the amino acid level, the three γ isoforms are over 90% homologous to each other, but share only 51-59% homology with other CKI family members in the catalytic domain. Also from rat testis, the full length cDNA encoding CKIδ, with a predicted molecular weight of 49.1 kDa, was first cloned (Graves et al., 1993). Three differently-sized messages of CKIδ were detected, with the largest message in all tissues tested and the smaller two predominantly expressed in testis. CKIε, with a predicted molecular weight of 47.3 kDa, was first identified from human placental cDNA library (Fish et al., 1995). It was known to be expressed in several human cell lines, but the tissue distribution of this isoform remains unclear. Among all vertebrate CKI isoforms, CKIε is closest to CKIδ in primary sequence, with greater than 98% identity at the amino acid level through their catalytic

domains. In addition, both CKIδ and CKIε possess a long carboxyl terminal tail, with 40% identity at the amino acid level, and with a similar regulatory function (discussed below). Although a long C-terminal domain is also found in each of the three γ isoforms, in particular γ-3, they do not share any significant homology to that of either δ or ε.

Yeast CKI Isoforms

Multiple isoforms of CKI have also been identified in yeast, including four isoforms in S. *cerevisiae* (Hrr25, Yck1, Yck2 and Yck3) ranging in size from 57 to 62 kDa, and five isoforms in S. *pomb* (Hhp1, Hhp2, Cki1, Cki2 and Cki3) ranging in size from 42 to 50 kDa. At the amino acid level, Hrr25 is related to Hhp1-2, as are Yck1-2 to Cki1-3. Both fission yeast Hhp proteins are functionally homologous to budding yeast Hrr25 protein because they all have been implicated in DNA repair and normal cell cycle progression, and yeast defective in these genes show hypersensitivity to DNA damage (Dhillon and Hoekstra, 1994; Hoekstra et al., 1991). YCK1 and YCK2 are an essential gene pair, and they, as well as Cki1 and Cki2, possess an isoprenylation motif at their carboxyl termini, thus encoding plasma membrane-associated CKI proteins. YCK1 was isolated as a suppressor of a defective SNF1 protein kinase activity, and YCK2 by its ability to relieve sensitivity of wild type cells to salt stress (Robinson et al., 1992; Wang et al., 1994; Wang et al., 1992). Yeast mutants deficient for these two genes display defects in cellular morphogenesis, cytokinesis, and endocytosis (Robinson et al., 1999; Robinson et al., 1992; Robinson et al., 1993). Unlike YCK1 and YCK2, the YCK3 gene product is predominantly associated with the nucleus, and Yck3p can weakly substitute for Yck1p-Yck2p (Wang et al., 1996b). So far, gene disruption studies have not identified

any essential function for the three highly related S. *pombe* CKI isoforms (Cki1, Cki2 and Cki3) (Kitamura and Yamashita, 1998).

From the view of evolution (Longenecker et al., 1996), yeast CKI isoforms are more homologous to the larger isoforms of vertebrate CKI isoforms. Cki/Yck subfamily is closer to CKIγ1, 2 and 3, whereas Hhp/Hrr25 subfamily is closer to CKIδ and CKIε. Functionally, yeast CKI genes overlap with their corresponding mammalian homologues. For example, CKIγ1 and CKIγ3 were found to complement defects in yeast cells defective in the YCK genes (Zhai et al., 1995). Similarly, expression of hCKIε but not hCKIα rescued the slow-growth phenotype of a S. *cerevisiae* strain with a deletion of HRR25 (Fish et al., 1995). It is noteworthy that neither phylogenetic studies nor functional analysis has suggested any yeast homologue for CKIα and CKIβ, implying that they may have envolved to carry out functions unique to higher eukaryotic systems.

Alternatively Spliced Isoforms of CKI

Among the seven CKI genes in vertebrates, α and γ3 genes are unique in that they can be alternatively spliced. Previously, our lab has identified as many as four CKIα transcripts in chicken: α, αS, αL and αLS (Green and Bennett, 1998), some of which were also found in cow (Rowles et al., 1991), rat (Zhang et al., 1996), human (Fish et al., 1995; Tapia et al., 1994) and frog (Pulgar et al., 1996). They differ only in the presence or absence of two inserts, L and S. The L insert, containing 28 amino acids, was first reported in bovine CKIαL, and then detected also in rat, chicken and human. The S insert, containing 12 amino acids, was first identified in human CKIα, and then was also observed in chicken and frog. The L insert is located in the kinase catalytic domain, whereas the S insert is located at the carboxyl terminus. Both the L and the S insert are

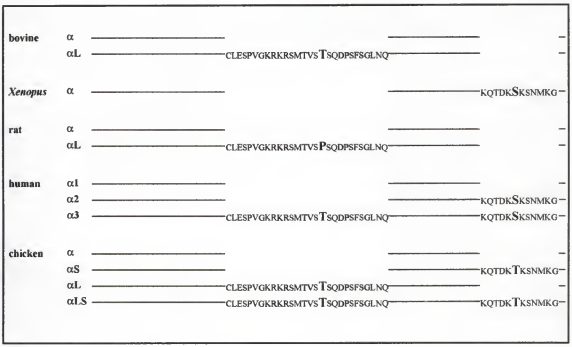


Figure 1-1. Diagram of vertebrate CKIα isoforms. All vertebrate CKIα isoforms across different species are 100% identical at the amino acid level, except for the presence or absence of two inserts, L and S. Both the L and the S inserts contain a conservative amino acid substitution (Green and Bennett, 1998).

100% identical at the amino acid level among different species, except that each contains one conservative amino acid substitution (Fig. 1-1). Genomic Southern analysis has suggested the presence of a single CKI alpha gene with two exons that are alternatively spliced (Green and Bennett, 1998). The CKIγ3 gene is also alternatively spliced within its carboxyl terminal end, resulting in two isoforms that differ in the presence or absence of a short 8 amino acid-insert, which shares no homology with either the L or the S insert at the amino acid level (Zhai et al., 1995). Sequence analysis has revealed that the L insert contains a strong putative nuclear localization signal, whereas neither the S insert nor the γ3 insert contains any known distinctive motif. In chapter 4, possible functions of the L and S inserts will be addressed experimentally.

Clearly, alternative splicing adds more complexity and more features to an already large CKI family. The elucidation of the functions of these inserts will help to clarify the differences among distinct isoforms.

Phosphorylation Recognition Motif for CKI

CKI was characterized as a serine / threonine protein kinase recognizing acidic residues in its substrates, therefore termed an acidotropic protein kinase (Flotow and Roach, 1991). Many lines of evidence collectively suggest the importance of Ser(P) as a determinant for CKI recognition, especially in the recognition motif Ser(P)-X-X-Ser/Thr (Flotow et al., 1990; Flotow and Roach, 1991). CKI's ability to utilize a phosphate group as a recognition determinant could link its activity to other kinases. For instance, prior phosphorylation of glycogen synthase by cAMP-dependent protein kinase is a

prerequisite for the phosphorylation of this substrate by CKI (Flotow and Roach, 1989; Zhang et al., 1993). Some effective CKI substrates, however, do not require prior phosphorylation. For example, inhibitor-2 of protein phosphatase 1 contains a cluster of acidic residues N-terminal to the target site (Pinna and Donella-Deana, 1994). In addition, a synthetic peptide substrate that contains 4 acidic residues N-terminal to the target site is also a relatively specific CKI substrate (Flotow and Roach, 1991). Taken together, the consensus sequence for CKI phosphorylation might be summarized as either $S(P)X_{1-3}S/T$ or $D/E_{2-4}X_{0-2}S/T$, with the former being more potent (Kennelly and Krebs, 1991). It should be mentioned that the chicken NF-M contains as many as 25 or probably 26 in vivo phosphorylation sites in the tail domain, and five of these match the CKI consensus sequence (discussed in chapter 3).

Recently, evidence emerged suggesting that CKI can be a dual specificity kinase in that it also phosphorylates tyrosine residues. It was shown that Hhp1, Hhp2 and Cki1 were able to autophosphorylate on tyrosine residues, and that Hhp1 and Hhp2 can also phosphorylate a synthetic peptide (poly-E_4Y_1) (Hoekstra et al., 1994). Also, *Xenopus* CKIα exhibited weak phosphorylation activity on polyE_4Y_1 peptide (Pulgar et al., 1996). The pool of potential in vivo CKI substrates has been significantly expanded due to the dual specificity of CKI. However, further investigation is required to establish any in vivo biological significance to the phosphorylation of tyrosine residues by CKI.

Expression, Tissue Distribution and Subcellular Localization

Expression and Tissue Distribution

Our knowledge about the expression and tissue distribution of different vertebrate CKI isoforms has been summarized in Table 1-1. At the message level, the α, γ-3 and δ isoforms exhibit wide tissue distribution, whereas the γ-1 and γ-2 isoforms are restricted to testis. As far as the α isoform is concerned, our lab has previously shown that the four alternatively spliced CKIα isoforms are expressed in many chicken cells and tissues (Green and Bennett, 1998). To date, the tissue distribution of the messages for the ε isoform is largely unknown, and also very little is known about the β isoform, except that its transcript is found in brain where it was originally cloned (Rowles et al., 1991).

Relatively little is known about the tissue distribution of CKI isoforms at the protein level. The combination of immunoprecipitation and western blotting experiments has previously demonstrated the presence of both CKIα and CKIαL at the protein level in all rat tissues examined (Zhang et al., 1996). Our data (presented in chapter 3) based on western blot indicate that the four CKIα isoforms, and CKIε, as well as other probable CKI isoforms are all present at the protein level in chicken brain, which is consistent with the report of the presence of the α, δ, and ε isoforms at the protein level in human brain (Ghoshal et al., 1999; Kuret et al., 1997).

Subcellular Localization

Although the CKI activity has been detected in several different intracellular compartments, very little is known about the subcellular localization of any given specific CKI isoform in vertebrates, in particular the larger CKI isoforms.

Table 1-1 Expression and Tissue Distribution of Vertebrate CKI Isoforms

Isotypes	Species	Tissues	mRNA	Protein
Alpha	bovine α, αL	brain, thymus	4.1 kb, 2.2 kb	36.7 kD*, 38.9 kD
	rat α, αL	testis, lung, liver, kidney spleen, heart, brain	cDNA from RT-PCR	37.6 kD*, 38.9 kD*
	chicken α, αS αL, αLS	brain	cDNA from RT-PCR	37.6 kD*, 38.9 kD* 40.6 kD*, 41.9 kD*
Beta	bovine	brain		38.7 kD
	rat	testis, brain, heart, kidney muscle, spleen	2.6 kb	
		testis, brain, heart	1.6 kb	
Gamma	bovine	brain		
	rat γ-1	testis	2.0 kb	43.0 kD
	γ-2	testis	1.5 kb, 2.4 kb	45.5 kD
	γ-3	testis, brain, kidney liver, lung, muscle	2.8 kb	49.7 kD
		testis, brain, kidney, lung	4.2 kb	
	human γ-2	testis	2.4 kb	45.5 kD
		pancreas, thymus, prostate testis, ovary, colon, heart, lung, spleen, liver, kidney	3.0 kb	
Delta	bovine	brain		
	rat	testis, kidney, spleen, lung heart, muscle, brain	3.5 kb	49.1 kD
		testis, heart (weak)	1.9 kb, 2.2 kb	
	human	brain		49.1 kD*
Epsilon	human	transformed cell lines	2.9 kb, 1.8 kb	47.3 kD
	chicken	brain		47.0 kD#

* The protein with a molecular mass similar to that predicted from the deduced amino acid sequence has been detected in corresponding tissues.

The protein has been detected by an isoform-specific antibody, but the sequence is not cloned yet.

The information about chicken CKI is based on the data obtained in our lab.

In 1990, the subcellular localization of CKI in HeRo-SV, a SV-40 transformed human glioblastoma cell line, was reported (Grankowski and Issinger, 1990). By using an anti-yeast CKI antiserum, which was shown to recognize a 45 kDa CKI band in yeast (Grankowski et al., 1987) and cross-react with a band at the similar position in HeRo-SV, immunofluorescence staining demonstrated the presence of the 45 kDa CKI in both the cytoplasm and the nucleolus. It is not known, however, to which specific mammalian or yeast CKI isoform this 45 kDa CKI band may correspond.

In the human erythrocyte, a 34-36 kDa CKI protein was found to associate with the plasma membrane, while a CKI protein with a similar molecular mass was found in the cytosol (Bazenet et al., 1990). Sequence comparison by two-dimensional peptide mapping revealed that the membrane-bound CKI and the cytosolic CKI in erythrocyte are very similar but not identical. Subsequently, the human erythrocyte cytosolic CKI was purified, and partial sequence analysis identified it as the α-CKI isoform, whereas the identity of the erythrocyte membrane-bound CKI remains unknown. Following the purification and the identification of the human erythrocyte 34 kDa α-CKI, polyclonal rabbit antibodies were raised against it and affinity purified. Western blotting indicated that the antibodies are highly specific, recognizing only a 34 kDa protein in lysates of all mammalian cells tested.

Immunofluorescence studies in mouse fibroblasts and CHO cells by use of this antibody demonstrated a cell cycle-dependent localization of the 34 kDa α-CKI (Brockman et al., 1992). In interphase, the α-CKI is localized to vesicular cytosolic structures and to the centrosome, and as cells enter mitosis, the α-CKI staining is shifted to mitotic spindle fibers and kinetochore fibers. Using the same antibody, it was observed

that α-CKI partially co-localizes with both Golgi and endoplasmic reticulum markers in non-neuronal cells and with synaptic vesicular markers in neurons (Gross et al., 1995). However, due to the absence of both the L and the S inserts in the human erythrocyte 34 kDa CKI sequence, it is likely that this antibody would recognize all four alternatively spliced CKI alpha isoforms. Besides, it was suggested that this antibody cross-reacts with β-CKI isoform (Gross et al., 1995). Given that all four α-CKI isoforms and the β-isoform are situated in the molecular mass range of 37-42 kDa, it is quite possible that the single band at about 36 kDa detected by this antibody may comprise more than one CKI isoform. Therefore, it is still unclear whether the different intracellular staining of this antibody reflects the varying localization of one single CKI species at about 36 kDa or the specific compartmentalization of different isoforms of CKI that very closely migrate on the western blot. In either case, it is crucial to distinguish among the four CKI alpha isoforms in their respective subcellular localization. In chapter 4, GFP fusion technology is used to directly visualize the subcellular distribution of the four CKI alpha isoforms in several cultured cell lines, and this method can be extended to the study of other CKI isoforms as well. The most apparent advantage of this method over that of using the antibodies that are not isoform-specific is that it is able to distinguish among different specific CKI isoforms in terms of the subcellular localization.

Compared with higher eukaryotes, more information has been obtained in yeast with regard to the subcellular distribution of a given specific isoform. For instance, the subcellular distribution of three CKI homologues, encoded by the YCK1, YCK2, and HRR25 genes in budding yeast, has been determined by subcellular fractionation (Vancura et al., 1994). Both epitope-tagged Yck proteins were primarily associated with

the plasma membrane fraction, and this association was shown to be mediated by the prenylation motif at the C-terminus of both proteins. The epitope-tagged Hrr25p, however, was found predominantly in the nuclear fraction, and only partially in the plasma membrane fraction. Similarly, subcellular fractionation was used to analyze the intracellular distributions of two CKI homologues, Cki1 and Cki2, in fission yeast (Wang et al., 1994). Both isoforms cofractionated mainly with the cytoplasm, and apparently being excluded from the nuclear fraction. Further purification on sucrose gradients and analysis for organelle-specific enzyme markers indicated that Cki1 cofractionates exclusively with a marker for plasma membrane, whereas Cki2 does not comigrate with any of the marker enzymes, but pellets at the bottom of the gradient, suggesting it may associate with particles of greater density than most membrane-bound organelles. It is worth noting that neither Cki1 nor Cki2 contains the prenylation motif at their C-terminus, suggesting an unidentified sequence may be responsible for the completely different localization of these two isoforms within the cytoplasm.

Substrates and Functions

The potential importance of CKI in biological functions has been suggested by the broad substrate specificity and the wide spread distribution of CKI in different tissues and in different subcellular compartments. Although there is a long list of in vitro substrates for CKI including metabolic proteins, membrane-associated receptors, adhesion molecules, cytoskeletal proteins, and transcriptional factors, very few of them have been proven to be in vivo substrates and functionally characterized in terms of the

physiological relevance. In addition, in very few cases has the identity of the CKI activity responsible for phosphorylating a given substrate been characterized. Most of the early studies used the following criteria to identify a kinase activity as the CKI-like activity: (a) similar molecular weight to CKI, (b) utilization of ATP but not GTP as phosphoryl donor, (c) sensitivity to heparin and a specific CKI inhibitor, CKI-7, [N-(2-amino-ethyl)-5-chloroisoquinoline-8-sulfonamide] (Chijiwa et al., 1989), (d) phosphorylation of casein and more specific CKI peptide substrates. Only in a few cases, has the CKI-like kinase been identified as a specific CKI isoform after purification and the subsequent amino acid sequence analysis. With the cloning of multiple CKI isoforms, more powerful tools have been developed to clarify the identity of a CKI-like kinase, including isoform-specific inhibitors, isoform-specific antibodies, and recombinant CKI proteins.

Membrane Receptors

A CKI-like kinase activity has been shown to associate with the p75 tumor necrosis factor (TNF) receptor, and the CKI specific inhibitor, CKI-7, markedly diminished the p75 phosphorylation in vivo and stimulated TNF-mediated signaling for apoptosis (Beyaert et al., 1995). The β-subunit of the insulin receptor was shown to be phosphorylated by a CKI-like kinase in vivo, but the significance of this to the insulin receptor-mediated signaling cascade is still under study (Rapuano and Rosen, 1991). Also, phospholipase C-coupled m3-muscarinic receptor can be phosphorylated in an agonist-sensitive manner by CKIα in vitro, suggesting that CKIα may provide an alternative signaling pathway from that of G-protein-coupled receptor kinase family for the stimulus-dependent phosphorylation of G-protein-coupled receptors (Tobin et al., 1997; Waugh et al., 1999). It has been shown that the recombinant CKIγ2 can

phosphorylate beta-PDGF (platelet-derived growth factor) receptor in vitro, and resulted in a remarkable inhibition of the receptor's tyrosine kinase activity, thus leading to receptor inactivation (Bioukar et al., 1999).

Cell-Cell Adhesion and Junction Proteins

The two larger polypeptides of the neural cell-adhesion molecule (N-CAM) were found to be phosphorylated by at least two protein kinases in vitro (Mackie et al., 1989). Based on their chromatographic behavior and substrate activity, they were suggested to correspond to GSK-3 and CKI. Although those in vitro sites have also been suggested to be phosphorylated to a low level in vivo, the biological significance remains unknown. The lens fiber cell-specific gap junction protein connexin49 is also an in vivo substrate for a membrane-associated CKI-like kinase, and an in-gel kinase assay associated this CKI-like activity in the lens membrane with a major 39 kDa species, the identity of which remains to be defined (Cheng and Louis, 1999).

Nuclear Substrates

A CKI-like activity was suggested to be involved in the phosphorylation of the cAMP response element modulator (CREM), thus enhancing the DNA-binding activity of this transcriptional factor (de Groot et al., 1993). A 35 kDa kinase, purified from HeLa cell nuclei, was shown to phosphorylate Simian virus 40 large T antigen at sites that are phosphorylated in vivo, thus causing the inhibition of SV40 DNA replication (Cegielska et al., 1994). On the basis of its molecular weight, substrate specificity, immunoreactivity and limited sequence analysis, this kinase is likely to be CKI, and probably one of the α isoforms.

The p53 tumor suppressor can also be phosphorylated at a group of sites on the N-terminus by CKI activity both in vitro and in vivo, and two specific isoforms CKIδ and CKIε were found to be responsible (Knippschild et al., 1997). In this study, a combination of a CKIδ/ε-specific inhibitor (IC261), a CKIδ-specific monoclonal antibody and recombinant CKIδ and CKIε proteins was utilized to disclose the isoform identity of this CKI-like kinase. However, the physiological significance of p53 phosphorylation by CKI is still not clear.

It has been known for many years that eukaryotic DNA-dependent RNA polymerase II can be phosphorylated by a CKI-like activity in vitro, although it is still elusive as to the biological significance of this phosphorylation in RNA polymerase II-mediated pre-mRNA synthesis (Dahmus, 1981). In addition, recombinant CKIα was shown to be capable of phosphorylating particular splicing factors within a region rich in serine/arginine dipeptide repeats, suggesting it may also be involved in regulating one or more steps of mRNA splicing (Gross et al., 1999).

Recently, a 40 kDa CKIα was reported to directly bind to and phosphorylate NF-AT transcriptional factor in vivo, thus inhibiting NF-AT nuclear import (Zhu et al., 1998). Due to the limited sequence information, it is not clear as to which one of the four α isoforms this 40 kDa CKI may correspond to.

Cytoskeletal Proteins

A CKI-like kinase, isolated from human erythrocyte cytosol, was able to catalyze the phosphorylation of a number of erythrocyte membrane-skeleton proteins, including spectrin and band-3 (Simkowski and Tao, 1980). Phosphorylation of beta-spectrin by membrane-bound CKI was implicated in the regulation of mechanical stability of the

erythrocyte membrane (Manno et al., 1995). Band 3 was shown to be phosphorylated at the cytoplasmic domain, primarily on Thr-42. Because Thr-42 is positioned near the binding sites for other membrane skeletal proteins, the phosphorylation of band-3 by CKI could conceivably be linked to the regulation of erythrocyte membrane structural integrity (Wang et al., 1997).

CKI (isoform identity not known) purified from bovine brain has been found to have the greatest ability to induce Alzheimer-like epitopes on the microtubule-associated protein tau, suggesting that CKI may play an important role in the conversion of τ from the normal to the abnormal phosphorylation state in Alzheimer disease (Singh et al., 1995). More recently, the tight association of CKIα and CKIαS with paired-helical filaments isolated from AD brains positioned these two specific CKI isoforms in the participation of hyperphosphorylation of protein tau (Kuret et al., 1997).

A CKI-like activity was also related to the phosphorylation of neurofilaments in the giant axon of the squid (Floyd et al., 1991). Similarly, our lab has previously detected a CKI-like activity that associates with chicken neurofilaments and selectively phosphorylates NF-M tail at several specific sites (Hollander and Bennett, 1992; Hollander et al., 1996). In chapter 3, I will discuss the identification of this neurofilament-associated and CKI-like kinase as two specific CKI isoforms, CKIα and CKIαS. The investigation of the potential functions of these CKI phosphorylation sites on NF-M tail are still underway.

Metabolic Enzymes

Concerted phosphorylation of rabbit muscle glycogen synthase by cyclic AMP-dependent protein kinase (PKC) and CKI inactivated the enzyme activity (Flotow and

Roach, 1989; Zhang et al., 1993). The phosphorylation of aminoacyl-tRNA synthetase complex from rabbit reticulocytes by CKI was able to alter the activity of these enzymes by the inhibition of their aminoacylation (Pendergast and Traugh, 1985). In both cases, the isoform identity of CKI is not clear.

Phosphatase Inhibitors

A CKI-like activity can also phosphorylate the dopamine- and cAMP-regulated phospho-protein of 32 kDa (DARPP-32), an inhibitor of calcineurin (the catalytic subunit of Ca^{2+}/calmodulin-dependent protein phosphatase 1c) (Desdouits et al., 1995). The phosphorylation of DARPP-32 by CKI was able to activate the inhibitor by blocking its dephosphorylation, thereby down-regulating the calcineurin activity. Similarly, inhibitor 2 of protein phosphatase 1 can be phosphorylated in vitro at Ser-86 and Ser-174 by a CKI-like activity, and the phosphorylation of Ser-174 was suggested to be detrimental to the activation of the phosphatase (Agostinis et al., 1992).

Signaling Molecules

By use of a specific antibody, CKIγ2 was shown to coimmunoprecipitate with Nck, a cytosolic adaptor protein mediating signal transduction initiated by receptor tyrosine kinase (RTK), suggesting that CKIγ2 may very well be involved in signaling pathways downstream of RTKs (Lussier and Larose, 1997). 14-3-3 proteins, adaptors mediating interactions between proteins involved in signal transduction and cell cycle regulation, can also be phosphorylated by CKIα at Thr-233 in vivo, and this phosphorylation negatively regulated the binding of 14-3-3 to c-Raf, thus impeding Raf-mediated signal transduction (Dubois et al., 1997).

The Drosophila clock gene double-time encodes a protein closely related to human CKIε, suggesting a role of CKIε in the regulation of mammalian circadian rhythms (Kloss et al., 1998). Also, CKIα was found to associate with synaptic vesicles and phosphorylate a specific subset of vesicle proteins, implicating a potential role of CKI in the regulation of vesicular trafficking and synaptic transmission (Gross et al., 1995). Due to the cell-cycle dependent localization to mitotic spindles, CKIα may be involved in the regulation of cell cycle progression, although no specific substrate of CKI for this link has been suggested (Brockman et al., 1992; Gross et al., 1997).

Very recently, both gain-of-function and loss-of-function studies have clearly linked CKI to the transduction of Wnt signaling pathway in *Xenopus* embryos (Peters et al., 1999). *Xenopus* CKIε, and probably the α isoform as well, was implicated to be involved through association and phosphorylation of dishevelled protein. In yeast, PtdIns(4)P 5-kinase was suggested to be a target of Cki in S. *pombe*, and the phosphorylation and inactivation of PtdIns(4)P 5-kinase by Cki is able to regulate PtdIns(4,5)P_2 synthesis (Vancurova et al., 1999).

Regulation of CKI Functions

In general, the regulation of CKI functions is still poorly understood. Only recently, has some evidence begun to emerge suggesting that autophosphorylation and isoform sorting may serve as two important means to regulate CKI functions in vivo.

Autophosphorylation

CKI has been known to be able to autophosphorylate for almost two decades (Dahmus, 1981), but not until recently has CKI autophosphorylation been linked to the regulation of the kinase catalytic activity of some isoforms with long C-terminal sequence.

In 1995, Graves et al. first presented evidence that the 110-aa long COOH-terminal domain of CKIδ has regulatory properties (Graves and Roach, 1995). They found that dephosphorylation of the C-terminal domain of CKIδ caused a progressive activation of the enzyme with a maximal activation of 2-3 fold. Further analysis of a series CKIδ C-terminal truncation mutants revealed an inhibitory region between His317 and Pro342 containing at least 6 potential phosphorylation sites, and removal of this region resulted in a nearly 10-fold increase in the specific activity of the enzyme. Similarly, limited tryptic digestion of the 123-aa long C-terminal extension of CKIε enhanced the core kinase activity by 7-fold, and dephosphorylation of the C-terminal tail significantly activated the kinase (Cegielska et al., 1998). In addition, it was reported that while both CKIδ and CKIε were able to autophosphorylate in vivo, they were actively maintained in the dephosphorylated state by cellular protein phosphatases (Rivers et al., 1998). Therefore, the autophosphorylation and dephosphorylation cycle on the C-terminal domain may serve as an essential regulatory mechanism in vivo for both CKIδ and CKIε. However, further study is required to determine as to how cellular protein phosphatases are regulated to either repress or stimulate the autophosphorylation of CKIδ and CKIε.

It should be pointed out that this autoinhibitory mechanism has not been reported in the smaller CKI isoforms (α and β). This is because unlike the long C-terminal tails of δ

(110 aa) and ε (124 aa), those of α (25 aa) and β (13 aa) are extremely short, and besides there is no significant homology in the amino acid sequence of the tails. Therefore, other mechanisms are likely required to regulate the α and β isoforms. As far as the α isoforms are concerned, the appearance of two inserted sequence, L and S, has prompted the speculation that they may be involved in the regulation of biological functions.

Isoform Sorting

The proposal that the specificity of kinase signaling is related to the targeting of different isoforms to specific substrates in different locations has been well demonstrated in PKC family. There are at least 11 different PKC isoforms, and following activation, most isoforms associate with the plasma membrane and some of them also translocate to specific locations including ER, Golgi, membrane ruffles, focal adhesions, cell-cell contact, perinuclear membrane and nuclear pores (Goodnight et al., 1995). So, although these PKC isoforms have similar enzymatic properties, differential subcellular compartment sorting suggests isoform-specific functions for at least some of them.

As detailed above, CKI is also widely distributed. Given that CKI is a large family, it is reasonable to speculate that this widespread distribution pattern is probably due to the presence of many distinct CKI isoforms and alternatively spliced forms. It is conceivable that the specific localization of each isoform is closely related to its function and regulation. For instance, it has been well documented that some yeast CKI isoforms (Yck 1 and Yck2) associate with the plasma membrane through the isoprenylation motif at their carboxyl-terminus (Vancura et al., 1994). This membrane-association appears very critical for their biological functions because once the isoprenylation motif was deleted, they dissociated from the plasma membrane and thus cannot function properly (Wang et

al., 1996b). No such isoprenylation motif has been identified in any mammalian CKI isoform yet.

Among all vertebrate CKI isoforms, CKIα is at present the best characterized in terms of the differential subcellular localization. Previously, it was reported that both the erythrocyte membrane-bound and the cytosolic CKI interact with native membranes and that this interaction is regulated by the membrane content of PIP_2 (Brockman and Anderson, 1991). Furthermore, the CKI activity on native membranes can be potently inhibited by a small increase (10-20%) in the membrane content of either exogenously added or endogenous PIP_2 (Brockman and Anderson, 1991). Taken together, it is suggested that the CKI activity can be regulated by PIP_2 when the kinase is associated with native membranes. However, the observation that incubation of PIP_2 with 1mM Mg^{2+} prevents the inactivation of the membrane-bound CKI by PIP_2 raises questions about the physiological significance of PIP_2-mediated inhibition of the CKI activity on native membranes (Chauhan et al., 1993). CKIα has also been detected in association with some cytoplasmic vesicular structures (Gross et al., 1995) and with mitotic spindles (Brockman et al., 1992). Although some in-vitro substrates have been suggested, it remains basically obscure as to what protein targets with which CKIα can interact at these specific locations to perform certain biological functions. In addition, given there are as many as four alternatively spliced CKIα isoforms, in neither case above have the four distinct CKIα isoforms been distinguished. Therefore, it is very important to know if different CKIα isoforms have the capacity of isoform-specific targeting to different subcellular compartments for different functions.

Summary

Casein Kinase I is a large and highly conserved serine/threonine protein kinase family. For a long time, CKI was mainly characterized based upon its molecular mass, isoelectric point and biochemical properties, such as the selectivity of acidic rather than basic protein substrates, the preference for ATP over GTP as the phosphoryl donor, and the specific inhibition of the activity by CKI-7. Following these criteria, a large number of substrates have been identified for CKI in vitro, but very few of them have been shown to be physiologically relevant. Only recently, with the cloning of different CKI isoforms, has it become apparent that CKI is not a single kinase, but a family with many distinct members. The question then arises as to which isoform(s) is responsible for the phosphorylation of a given substrate in vitro as well as in vivo. Meanwhile, since CKI activity has been detected in many different intracellular locations, a related question is whether or not different isoforms have differential compartmentalization.

Among all vertebrate CKI isoforms, CKIα is the best characterized in terms of its subcellular localization and potential functions. It has been found in the cytosol and membrane, associating with vesicular structures, mitotic spindles, and nuclear structures. Also, a number of in vitro substrates for CKIα have been suggested. However, due to the presence of as many as four alternatively spliced CKIα isoforms, it remains to be determined if the four isoforms reside in all these intracellular sites, or if they may display selective distribution to different locations. In addition, it remains to be determined as to which isoform(s) is responsible in a specific cellular event.

During the study of neurofilament phosphorylation, we detected a neurofilament-associated CKI-like kinase activity (NFAK) that selectively phosphorylates several specific serine residues in the chicken NF-M tail (Hollander and Bennett, 1992; Hollander et al., 1996; Shaw et al., 1997). Meanwhile, we were able to identify four alternatively spliced CKIα isoforms from chicken brain cDNA library (Green and Bennett, 1998). Further characterization of NFAK revealed that it is none other than two specific CKIα isoforms (discussed in chapter 3). Subsequently, we studied the localization of the four isoforms, and found that they are differentially compartmentalized (discussed in chapter 4). In summary, our study presented here is focused on the characterization of these four CKIα isoforms, with the aim of trying to elucidate the differences in their localization and function.

CHAPTER 2
MATERIALS AND METHODS

Materials

Frozen chicken brains were purchased from Pel-Freez Biologicals (Rogers, AR, USA), glutathione-Sepharose from Pharmacia Biotech (Piscataway, NJ, USA), protein A-agarose from Boehringer Mannheim (Indianapolis, IN, USA), and α-casein-agarose from Sigma (St. Louis, MO, USA). Also from Sigma were α-casein and phosvitin. [γ-^{32}P]ATP was from ICN (Costa Mesa, CA, USA). ECL western blotting detection reagents were from Amersham Life Science (Arlington Heights, IL, USA). Immobilon-P membrane was from Millipore (Bedford, MA, USA). The monoclonal antibody against CKIε was from Transduction Laboratories (Lexington, KY, USA). Goat serum, fetal bovine serum, trypsin-EDTA, antibiotics Penicillin-Streptomycin, antimycotics Fungizone and lipofectamine were all from GibcoBRL Life Technologies (Grand Island, NY, USA). Affi-Gel 10 was from Bio-Rad (Hercules, CA, USA). Vectashield Mounting Medium containing DAPI was from Vector Technologies (Burlingame, CA, USA). The monoclonal antibody Y-12 anti-Sm was from Lab Vision Corporation (Fremont, CA, USA). The Concanavalin-A Alexa Fluor™ 594 conjugate was from Molecular Probes (Eugene, OR, USA), while the Wheat Germ Agglutinin was from Sigma (St. Louis, MO, USA). EnzyPlus polymerase was from EnzyPol Ltd (Denver, CO, USA). Immunoaffinity-purified polyclonal CKI antibody was from StressGen Biotechnologies

(Victoria BC, Canada). Monoclonal Green Fluorescent Protein (GFP) antibody was from CHEMICON International, Inc. (Temecula, CA, USA).

Methods

Purification of a Neurofilament-Associated Kinase (NFAK)

The procedure used for preparing high-salt extract containing NFAK activity was the same as that detailed previously (Hollander and Bennett, 1992), except that the starting material was a chicken brain detergent-insoluble cytoskeletal preparation (Shaw et al., 1997). In brief, chicken brains were homogenized in buffer A [10 mM Tris, 5 mM EGTA, and 2 mM DTT, pH 7.4] and centrifuged at 11,000 g for 20 min. The pellet was then resuspended in buffer B (buffer A plus 0.5% Triton X-100) and again centrifuged. This was repeated twice. The final pellet was resuspended in 0.6 M KCl in buffer B and stirred in the cold overnight. The suspension was briefly centrifuged at 11,000 g, and the supernatant was clarified by centrifugation at 110,000 g for 1 h. The resulting high-salt extract was then dialyzed against MES buffer [50 mM MES (pH 6.5), 10 mM $MgCl_2$, 1 mM EDTA, 2 mM EGTA, 1 mM DTT, and 5% glycerol] and applied to a DEAE-cellulose column that was directly connected to a phosphocellulose column. Both columns were preequilibrated with MES buffer. This system was washed with MES buffer until $O.D._{280}$ of the eluant reached almost zero. The DEAE column was then disconnected, and the phosphocellulose column was eluted with 0-0.5 M NaCl gradient in MES buffer. Fractions were assayed for NFAK activity (see below), and active fractions were pooled, dialyzed against Tris buffer [50 mM Tris (pH 7.2), 1 mM EGTA, 1 mM

DTT and 5% glycerol], and concentrated with an Amicon YM-10 membrane. The phosphocellulose fraction (30 mg) was then applied to a Sephacryl S-200 column (300 ml) in 0.5 M NaCl in Tris buffer. Fractions containing NFAK activity were pooled, concentrated and desalted on a YM-10 membrane. The Sephacryl fraction (2 mg) was mixed with 2 ml of casein-agarose beads, poured into a column, washed extensively, and developed with a 0-0.6 M NaCl gradient. Fractions within the NFAK activity peak were pooled, concentrated, and stored at -70°C in Tris buffer with 20% glycerol. Included throughout the purification was a cocktail of protease inhibitors at the following concentrations: 10 μg/ml leupeptin, 2 μg/ml pepstatin, 0.25 mM phenylmethylsulfonyl fluoride, and 3 μg/ml aprotinin.

In Vitro Kinase Assay

The reaction mixture contained 25 mM Tris (pH 7.5), 5 mM $MgCl_2$, 10 mM DTT, 4X protease inhibitor cocktail, 20 μM [γ-^{32}P]ATP (0.5 μCi), 1-30 μl of kinase sample, and 2-5μg of exogenous substrate in a final volume of 60 μl. After incubating at 37°C for 20 min, the reaction mixture was precipitated in cold acetone and collected by centrifugation. The pellet was dissolved in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (Laemmli, 1970) and separated on a 10% SDS gel. The dried gel was exposed for autoradiography or scanned and quantitated on a PhosphorImager (Molecular Dynamics).

Casein and phosvitin were used as substrates to assay casein kinase activity, and the recombinant protein encoded by CM:381-558 plasmid was used as a specific substrate for NFAK activity. The CM:381-558 plasmid encodes a fusion protein containing 324 amino acids of the *Escherichia coli trp*E gene and a 178-amino acid sequence from chicken NF-

M, which includes all NFAK/CKI sites but no Ser/ThrPro sites (Fig. 3-1). The construct was expressed and purified as described (Hollander et al., 1996).

In-Gel Kinase Assay

The protocol for in situ renaturation and assay of NFAK activity after SDS-PAGE was derived from that described by (Geahlen and Harrison, 1986 and Hutchcroft, 1991 #86). NFAK samples were loaded onto 10% SDS gels polymerized in the presence of either 1 mg/ml phosvitin or 2 mg/ml CM:381-558 fusion protein. After electrophoresis, gels were subjected to denaturation and renaturation treatments as described (Hutchcroft et al., 1991). For detection of kinase activity, the gel was incubated in 20 ml of kinase buffer [50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, and 20% glycerol] at 4°C for 30 min and then incubated with kinase buffer plus 20 μM ATP and 100 μCi of [γ-^{32}P]ATP at 37°C for 2 hr. After termination of the reaction with 5% trichloroacetic acid containing 1% sodium pyrophosphate, the gel was exposed for autoradiography and PhosphorImager analysis.

Preparation of Casein-binding Proteins

About 1 g of frozen chicken brain tissue was homogenized in 2.5 ml of buffer B plus protease inhibitors and centrifuged at maximal speed in a microcentrifuge for 20 min. The supernatant was then incubated with 25 μl of casein-agarose beads at 4°C for 30 min. After extensive washing, beads were incubated with SDS sample buffer and heated at 95°C for 5 min.

CKIα Antibodies

The L insert encoding amino acids 153-180 (CLESPVGKRKRSMTVSTSQDPSFSG LNQ) and a sequence common to all four cCKIα isoforms encoding amino acids 183-249 (LIDFGLAKKYRDNRTRQHIPYREDKNLTGTARYASINAHLGIEQSRRDDMESLG YVLMYFNRTSLPW) were obtained by Polymerase Chain Reaction (PCR) from a plasmid containing the full-length CKIαL insert (Green and Bennett, 1998). They were first ligated into pT7-blue (Novagen), cut out, and ligated into pGEX4T-2 (Amersham Pharmacia Biotech) for expressing glutathione S-transferase (GST) fusion proteins. The S insert encoding amino acids 353-364 (KQTDKTKSNMK) was synthesized and directly cloned into pGEX4T-2. All three constructs were transformed into E. *coli* NM522. GST-L and GST-S were expressed and affinity-purified on glutathione-agarose according to manufacturer's instructions. GST-C, however, was restricted to inclusion bodies and required 0.2% Sarcosyl for solubilization. Once bound to glutathione beads it could not be removed with standard procedures. Therefore, GST-C was stripped with SDS and gel-purified.

Rabbits were immunized with each fusion protein, and the resulting antisera were affinity-purified. The antisera were first applied to a column of GST coupled to Affi-Gel 10 for removal of anti-GST antibodies, followed by a column of the appropriate fusion protein also coupled to Affi-Gel 10. Purified antibodies were stored in the presence of 5% glycerol at -70°C. They are designated anti-L, anti-S, and anti-C.

Anti-CKI peptide is a polyclonal antibody to CKI that was kindly provided by Dr. Jorge E. Allende. It was raised against the synthetic peptide ^{147}GRHCNK^{152} ^{181}LFLIDF GLAKKY^{192} (Pulgar et al., 1996).

Western Blotting

Western blotting was basically carried out according to the ECL western blotting analysis protocol (Amersham Life Science). After electrophoresis and transfer, the membrane was blocked in phosphate-buffered saline containing Tween plus 6% goat serum and then incubated for 1-2 h with primary antibodies (0.2-1.0 μg/ml affinity-purified polyclonal antibodies or 1:2,000-1:4,000 dilution of polyclonal antiserum). Peroxidase-coupled secondary antibodies were applied at a 1:10,000 dilution for 45 min, followed by ECL reagents. Chemiluminescence signals were captured on FUJI medical x-ray film.

Immunoprecipitation of NFAK

Protein A-agarose was incubated with either affinity-purified antibodies or polyclonal antiserum in Tris buffer [50 mM Tris (pH 7.2), 1 mM EGTA, 1 mM DTT, and 5% glycerol] plus protease inhibitors at 4°C for 1 h and then washed extensively to remove nonbound proteins. Subsequently, coated protein A-agarose beads were incubated with purified NFAK samples at 4°C for 1 h. Supernatants were separated from beads by brief centrifugation. The beads were extensively washed in Tris buffer plus 0.5 M NaCl and 0.1% Nonidet P-40 and finally resuspended in the original volume of Tris buffer. Equal aliquots of supernatants and resuspended beads were subjected to in vitro kinase assay for determining NFAK activity.

Cloning of GST-CKI and GFP-CKI Fusion Constructs

Previously each of the four chicken CKIα full-length cDNAs was cloned into pT7Blue (Green and Bennett, 1998). Among all those clones subjected to sequencing, most of them contained a few random non-silent errors probably due to the use of the Taq

polymerase. Only one clone (pCL23-4, CKIαLS) was obtained which encoded perfect amino-acid sequence. From this clone, the full length cDNA sequences for CKIα (978bp), CKIαS (1014bp), CKIαL (1062bp) and CKIαLS (1098bp) were then generated and PCR amplified by using Splicing by Overlap Extension (SOE) (Horton et al., 1989). During the SOE, a high-fidelity polymerase EnzyPlus was used, and a 5'-BamHI site and a 3'-SalI site were introduced within the primers, as follows:

P1: 5'-GTCGGATCCCATA^{1}**TG**GCGAGCAGCAGCG^{16}-3';

P2: 5'-G^{432}GGTATTGGGCGTCACTGTAATAAGT^{457} T^{542}ATTCCTTATTGAC TTTGG^{560}-3';

P3: 5'-G^{439}GGCGTCACTGTAATAAGT^{457}T^{542}ATTCCTTATTGACTTTGGTTT GGCC^{567}-3';

P4: 5'-C^{1042}CAAACCCCCACAGG^{1055}T^{1092}TTC**TGA**^{1098}GTCGACTGAG-3';

P5: 5'-C^{1073}CAAGAGTAACATGAAAGGTTTC**TGA**^{1098}GTCGACTGAG-3'.

Primer 1 and 4 were used for producing CKIαL. Primer 1 and 5 were used for producing CKIαLS. To assemble CKIα, primer pairs 1-2 and 3-4 were used for primary PCR, and primer 1 and 4 for secondary PCR. Similarly, to assemble CKIαS, primer pairs 1-2 and primer 3-5 were used for primary PCR, and primer 1 and 5 for secondary PCR. The SOE products were then ligated directly into pGEX-4T-2 at EcoRI and BamHI sites, and transformed into E.*coli* DH5α. All DNA constructs were checked by sequencing. The GST-CKI fusion proteins were subsequently expressed in E.*coli* DH5α and affinity-purified on glutathione-agarose according to manufacturer's instructions.

For the purpose of cloning into a Green Fluorescent Protein (GFP) vector (pCI-Neo-eGFP, see below), the pGEX-CKI plasmids encoding four isoforms were chosen as

templates. To introduce a 5' EcoRI site and a 3' SalI site for cloning purpose, forward primer 5'-GTCGAATTC**A**[1]**TG**GCGAGCAGCAGCGGCTCC[21]-3' and reverse primers P4 (for α and αL) and P5 (for αS and αLS) were used. The PCR products were first ligated into pT7Blue vectors, cut out by SalI and EcoRI, and subsequently ligated into pCI-Neo-eGFP, and transformed into E.*coli* DH5α. The ligation products were sequenced and verified to be correct and in-frame. Subsequently, large quantities of high-quality plasmid DNA for each fusion construct were purified by centrifugation to equilibrium in Cesium Chloride-Ethidium Bromide gradients twice (Maniatis et al., 1982).

In our study, a modified version of eukaryotic expression vector (pCI-Neo-eGFP) was used to express GFP-CKI fusion proteins. The original version of this vector, pCI-Neo-GFP, described in detail previously (Wang et al., 1996a), contains a Ser65Thr mutation in the GFP sequence, which was shown to greatly increase the fluorescent signal (Heim et al., 1995). The current version of this vector is identical to pCI-Neo-GFP except that it contains a Phe64Leu mutation in the GFP sequence known to further increase the fluorescent signal (Tsien, 1998).

Cell Culture and Transient Transfection

COS-7, NIH-3T3 and LLC-PK cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, antibiotics (Penicillin-Streptomycin) and antimycotics (Fungizone). Transfections were carried out using Lipofectamine (GibcoBRL) following the guidelines supplied by the manufacturers. Briefly, cells were exposed to lipid/DNA complexes for 10-12 hours at 37°C in serum-free DMEM medium after they had reached 30-40% confluence on glass coverslips in

35-mm culture dishes. Subsequently, cells were incubated at 37°C in DMEM with 10% fetal bovine serum for another 18-24 hours before they were fixed and examined by fluorescence microscopy.

Preparation of Cell Lysate containing GFP Fusion Proteins

Untreated COS-7 cells or COS-7 cells transfected with either GFP-CKIα or GFP-CKIαS were harvested and briefly washed in cold PBS (pH 7.4), and then resuspended in lysis buffer [PBS (pH 7.4), 0.2% Triton X-100, 2mM DTT and protease inhibitors] for 10 minutes. After pulse sonication, the cell lysate was centrifuged at 14,000g for 10 minutes, and both the supernatant and the pellet fractions were saved, incubated with SDS sample buffer and heated at 95°C for 5 minutes.

Fluorescence and Histochemistry

For visualizing GFP signals, transfected cells were rinsed in PBS, and then either fixed in 4% formaldehyde for 15 minutes at room temperature or preextracted with 0.2% Triton X-100 in PBS on ice for 2 minutes (Misteli and Spector, 1996) followed by fixation in 4% formaldehyde for 15 minutes at room temperature. After fixation, cells were rinsed in PBS and coverslips were mounted on slides using Vectashield Mounting Medium containing DAPI.

For co-localization studies with nuclear speckles, GFP-CKIαL or GFP-CKIαLS transfected cells were rinsed, pre-extracted, and fixed as described above. After permeabilization in 0.5% Triton X-100 for 5 minutes, cells were incubated in blocking buffer (3% goat serum, 0.1% Tween-20, PBS, pH 7.2) for an hour. The monoclonal Y-12 anti-Sm antibody recognizing splicesome snRNPs (small nuclear ribonucleoproteins) was then applied to cells on coverslips at 1:100 dilution for one hour, which was followed by

incubation with rhodamine-conjugated goat anti-mouse secondary antibody at 1:50 dilution for one hour. For the study of transcriptional inhibition, transfected cells on coverslips were incubated with 20 μg/ml amanitin for 3 hours before pre-extraction and fixation. All buffers were supplemented with 2mM $MgCl_2$.

For co-localization studies with the Golgi and the endoplasmic reticulum markers, GFP-CKIα and GFP-CKIαS transfected cells were briefly rinsed in 1XPBS buffer at room temperature, and then fixed in –20°C methanol for 10 minutes. After fixation, cells were incubated in blocking buffer for one hour, and then incubated with either rhodamine-conjugated wheat germ agglutinin (WGA) at 2.5 μg/ml or Alexa Fluor 594-conjugated concanavalin A (ConA) at 1 μg/ml for one hour.

Cells were observed with a Zeiss Axiophot (Carl Zeiss, Inc.) equipped with a SpotCam (Diagnostics, Inc.).

CHAPTER 3
IDENTIFICATION OF CKIα AND CKIαS AS A NEUROFILAMENT-ASSOCIATED KINASE

Introduction

Neurofilaments

The cytoskeleton is composed of three major components: actin filaments (6nm), intermediate filaments (8-10nm) and microtubules (24nm). Intermediate filaments, the major components in the cytoplasm of most eukaryotic cells, are believed to play key roles in cell architecture, stability and differentiation.

The current family of mammalian neuronal intermediate filaments consists of six members: the three neurofilament (NF) subunits NF-L (66kDa), NF-M (95-100kDa) and NF-H (110-115kDa), α-internexin (~60kDa), peripherin (~50kDa) and nestin (~200kDa) (Lee and Cleveland, 1996). All six members share a characteristic ~310 amino acid α-helical domain containing a hydrophobic heptad repeat essential for assembly, and the globular head and tail domains flanking this central rod domain which are markedly divergent in length and sequence. Being abundant in most mature mammalian neurons, neurofilaments are extraordinarily stable at neutral pH and physiological ionic strength. Many lines of evidence collectively indicate that NFs in vivo are obligate heteropolymers requiring NF-L and either NF-M or NF-H for assembly (Lee et al., 1993; Nakagawa et al., 1995).

Neurofilament Phosphorylation and Potential Functions

The most distinctive feature of the NF subunits, particularly NF-M and NF-H, are their long and heavily phosphorylated carboxyl-terminal tail domains. The NF-L tail is highly acidic with many glutamic acid residues comprising a segment referred to as the E segment (Harris et al., 1991). For NF-M, this tail region is longer and contains the E segment as well as segments rich in glutamic acid and lysine. NF-H is noted for the presence of between 42 and 51 lysine-serine-proline (KSP) repeats, among which the serine residues are always heavily phosphorylated in axons (Carden et al., 1985; Julien and Mushynski, 1982; Lee et al., 1988). NF-M also contains a few KSP motifs in which the serine residues are phosphorylated in axons, but their numbers and positions are not conserved (Harris et al., 1991). Meanwhile, some attention is also being increasingly drawn to the amino-terminal head domain. At least 6 distinct phosphorylation sites were found on the head domain of NF-M, and at least 2 on the head domain of NF-L (Sihag and Nixon, 1989; Sihag and Nixon, 1990).

In 1982, Lazarides first proposed that the assembly and functional properties of intermediate filaments may be regulated by phosphorylation (Lazarides, 1982). The phosphorylation of NF head domains has been suggested to be able to modulate NF assembly. For instance, deletions studies have shown that the head and rod, but not the tail, are essential for assembly (Chin et al., 1991; Gill et al., 1990; Lee et al., 1993; Wong and Cleveland, 1990). Also, phosphorylation of the head domains of NF subunits as well as vimentin and lamins are known to either inhibit assembly or cause disassembly of filaments both in vivo and in vitro (Ando et al., 1989; Hisanaga et al., 1990; Hisanaga et al., 1994; Inagaki et al., 1989; Sihag and Nixon, 1990).

Compared with other intermediate filaments, neurofilaments have unique "side arms" (composed of NF-M and NF-H tails) which protrude from the filament backbone and appear as cross-bridges in the electron microscope (Hirokawa et al., 1984; Hisanaga and Hirokawa, 1988). Conceivably, electrostatic repulsions between NF side arms due to the presence of many phosphates in the tail increase the filament spacing (Nixon and Sihag, 1991; Shaw et al., 1986). This hypothesis has been supported by several lines of circumstantial evidence. For instance, myelin-deficient axons have significantly reduced caliber, decreased NF spacing, and decreased levels of NF phosphorylation compared with normally myelinated segments (de Waegh et al., 1992). Similarly, axons diameter, NF spacing and level of phosphorylation are all less in axon initial segments and in nodes of Ranvier than in internodes (Hsieh et al., 1994). Furthermore, it has been proposed that phosphorylation-induced extention of carboxyl-terminal domain side arms may create a steric drag to slow axonal transport of neurofilaments (Nixon and Sihag, 1991). Also, it has been suggested that phosphorylation may increase NF stability during axonal growth and confer resistance to proteolysis intraaxonally (Pant, 1988).

Neurofilament Kinases

In order to fully understand how the phosphorylation modulates NF behavior, considerable efforts have been directed toward the identification of relevant kinases. Second-messenger-dependent protein kinases (PKC and PKA) have been reported to phosphorylate the head domain of NF subunits (Dosemeci and Pant, 1992; Hisanaga et al., 1994). In contrast, many second-messenger-independent protein kinases have been shown to phosphorylate NF at the carboxyl-terminal end. For instance, glycogen synthase kinase-3 (Guan et al., 1991; Guidato et al., 1996; Yang et al., 1995), extracellular signal-

regulated kinase (Roder and Ingram, 1991; Veeranna et al., 1998), members of the cyclin-dependent kinase family (Guidato et al., 1996; Hisanaga et al., 1991; Lew et al., 1992; Shetty et al., 1993; Sun et al., 1996), and stress-activated protein kinase γ (Giasson and Mushynski, 1997) have all been implicated in the phophorylation of NF-H and/or NF-M tails at multiple Ser-Pro sites.

NF-M Tail Phosphorylation and Casein Kinase I

Several KSP, KXSP and KXX(S/T)P motifs, along with a single KSD sequence, have been identified as in vivo phosphorylation sites in the C-terminal tail domains of porcine, rat, chicken, and bovine NF-M and NF-H (Bennett et al., 1994; Geisler et al., 1987; Xu et al., 1992; Yang et al., 1995). The segregation of separate phosphorylation motifs to different segments within the tail is most prominent in NF-M and is especially pronounced in chicken NF-M (Harris et al., 1991). So far, two distinct functions of the carboxyl-terminal tail domain of NF-M upon neurofilament assembly have been suggested: cross-bridge formation and longitudinal elongation of filaments (Nakagawa et al., 1995), but still little is known about how phosphorylation of NF-M tail at those specific sites contribute to these functions. Therefore, research interests of our lab have been focused on the characterization of chicken NF-M tail phosphorylation sites and kinase(s) involved.

Previously, it was shown that the bovine neurofilament-enriched preparation contains the kinase activity, which is similar to CKI, and phosphorylates all NF subunits predominantly at their carboxyl-terminal domains (Link et al., 1993). In contrast, our lab identified a kinase activity from the chicken neurofilament-enriched preparation that can selectively phosphorylate the chicken NF-M tail domain at several specific residues, and

this kinase activity is very similar to CKI (Hollander and Bennett, 1992; Hollander et al., 1996; Shaw et al., 1997).

NFAK – a Neurofilament-Associated Kinase

Although Ser/Thr-Pro sites are the most numerous in both NF-M and NF-H tails, there are additional phosphoserines in the NF-M tail that are not associated with a nearby proline. Previously, five such sites in the acidic amino-terminal portion of the chicken NF-M tail have been identified to be in vivo phosphorylation sites (Ser^{464}, Ser^{471}, Ser^{502}, Ser^{528} and Ser^{536}) (Fig. 3-1) (Hollander et al., 1996; Shaw et al., 1997). At least three of these sites are conserved in the corresponding mammalian sequences (Shaw et al., 1997). These sites all share a casein kinase I (CKI) consensus motif ($D/E_{2-4}X_{0-2}S/T$), and they can be phosphorylated in vitro by pure CKI (Hollander et al., 1996; Shaw et al., 1997) and a chicken NF-associated kinase (NFAK) (Hollander and Bennett, 1992). NFAK was initially identified in an NF-enriched preparation as cosedimenting with NFs in the presence of Triton X-100 but dissociable with high salt concentrations (Hollander and Bennett, 1992). After partial purification, the activity of NFAK was found to be CKI-like in that (a) NFAK activity can be inhibited by CKI-7, a selective CKI inhibitor, and (b) NFAK phosphorylates only those sites in NF-M that can be phosphorylated by pure CKI (Hollander et al., 1996; Shaw et al., 1997). Given that CKI is a large family, the question thus arises as to whether NFAK may correspond to all, or a subset, of the CKI family.

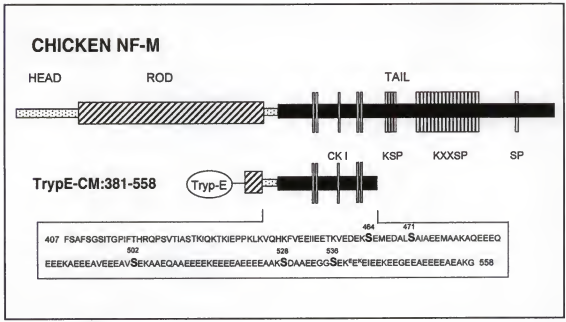


Figure 3-1. Diagram of chicken NF-M [modified from Bennett and Quintana (1997)] and the CM:381-558 fusion protein. Several distinct phosphorylation motifs are contained in the tail domain of chicken NF-M, including 5 CKI sites, 4 KSP repeats, 17 KXX(S/T)P repeats, and one SP site. The CM:381-558 fusion protein contains amino acid 381-558, beginning with the end of coil 2 and extending into the amino-terminal portion of the tail, including all five NFAK/CKI sites (expanded below) but none of the S/TP sites.

Experimental Results

Purification of NFAK

Previously, NFAK was partially purified from spinal cord NFs by DEAE-cellulose and phosphocellulose chromatography (Hollander and Bennett, 1992). To identify NFAK more precisely, we attempted to prepare larger amounts of more highly purified material. TrpE-CM:381-558 fusion protein (detailed in Methods) containing exclusively the five CKI/NFAK sites was used as the specific NFAK substrate during the purification (Fig. 3-1) Two additional steps, gel filtration on Sephacryl S-200 and casein-agarose affinity chromatography, were added (see Methods), which together resulted in an increase in the NFAK specific activity by another ~50 fold over what had been achieved previously (see Table 3-1). However, inclusion of further steps (either Cibacron Blue 3GA-agarose or hydroxylapatite chromatography) was accompanied by a drastic loss of kinase activity. Ultimately, a highly purified NFAK sample was obtained for further biochemical and immunological analysis to clarify its final identity.

Co-purification of NFAK and Casein Kinase Activities

During the last two steps of purification, kinase activity towards CM:381-558 fusion protein which contains the specific sites in NF-M, as well as activity towards two standard in vitro substrates for casein kinases: casein and phosvitin, was assayed. The activity profiles obtained with all three substrates coincided exactly for both the gel filtration and casein-agarose column (Fig. 3-2). Calibration of the Sephacryl gel filtration column with molecular weight standards placed the peak of CKI/NFAK at ~35-40kDa. A similar size was revealed when the Sephacryl NFAK fraction was applied to an in-gel

Table 3-1. Purification of NFAK

Sample	Protein (mg)	Kinase Activity (units*)	Sample Volume (ml)	Specific Acitivity (units*/mg)	Purification (fold)
DHSS	950	2.0	250	2.1	1
PC	30	0.8	6.5	26	12
SE	2.0	0.3	4.5	150	70
CA	0.18	0.24	2.2	1222	634

Note: DHSS = Dialysed High Salt Supernatant
PC = Phosphocellulose sample
SE = Sephacryl sample
CA = Casein Agarose sample

* based on phosphorimager units

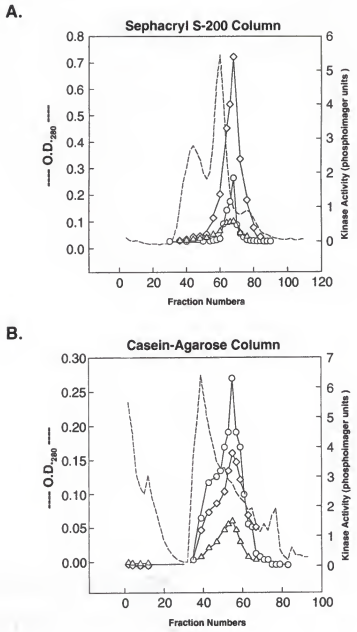


Figure 3-2. Copurification of NFAK and casein kinase activities. **A:** Gel-Filtration. Phosphocellulose-purified NFAK (see Methods) was applied to a Sephacryl S-200 column. Fractions were assayed for NFAK activity using the CM:381-558 fusion protein (◇), and for casein kinase activity using casein (○) and phosvitin (△). **B:** Casein-agarose affinity chromatography. The pooled Sephacryl fractions (A, 64-70) were applied to a casein-agarose column. Fractions were assayed for NFAK and casein kinase activities as in A.

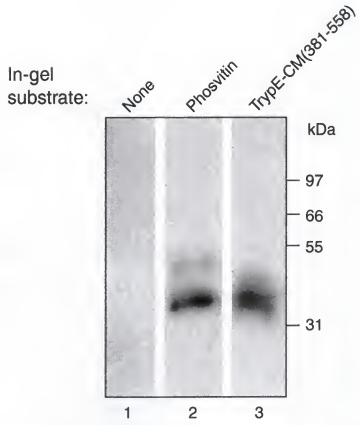


Figure 3-3. In-gel Kinase Assay. Sephacryl-purified NFAK (Fig. 3-2) was applied to an SDS-Gel containing either no substrate (lane 1), 1mg/ml phosvitin (lane 2), or 2mg/ml CM:381-558 (lane 3). After electrophoresis, proteins were denatured and then renatured in-gel, followed by incubation in kinase assay buffer with γ-32P ATP. The gel was stained, dried and subjected to phosphorimager analysis, shown here.

renaturation kinase assay (Fig. 3-3). Clearly, both phosvitin and TrpE-CM:381-558 revealed a strong phosphorylation band around 36-40kDa, whereas no band was detected in the absence of substrate. Phosvitin, but not CM:381-558, produced an additional, faint band at 45-50kDa. Taken together, the copurification and in-gel kinase data suggest that the component of NFAK that represents the major activity toward CM:381-558 is a casein kinase(s) of 36-40kDa. Given that isoforms of CKI family range in size from 36-39kDa for CKIα and CKIβ to 45-51kDa for CKIγ, CKIδ and CKIε, CKIα or possibly CKIβ was a reasonable candidate for NFAK. Since CKIα is not a single kinase, but consists of as many as four alternatively spliced isoforms, the question then arises as to which isoform(s)of CKIα, if any, may be responsible for the NFAK activity.

Detection of CKI Isoforms in Chicken Brain

As detailed in chapter I, vertebrate CKI alpha gene can be alternatively spliced, and up to four spliced variants have been identified in several species (Fig. 1-2). Only in chicken, however, have transcripts for all four isoforms been found. The only difference between the four alpha isoforms lies in two inserts: L and S. To address which one or more of these isoforms are present in chicken neuronal tissue at the protein level and whether they represent NFAK activity, three polyclonal antibodies which are selective for the L and S sequences as well as an immunogen sequence (C sequence) common to all four isoforms were raised (Fig 3-4; see also Fig. 1-1 and Methods). The C sequence, containing 67 amino acids, is identical in all four CKIα isoforms and also has many stretches of identity to other members of the vertebrate CKI family. On the contrary, neither the L nor the S sequence is found in other known proteins, as determined by a BLAST search (Altschul et al., 1990). All three antibodies were affinity-purified and the

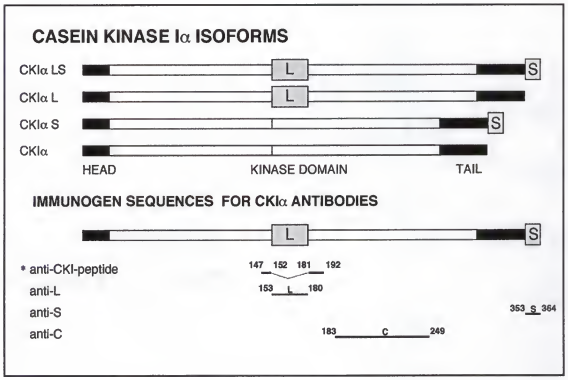


Figure 3-4. Diagram of four CKIα isoforms and location of sequences used for antibody production. Note that the only difference between the four chicken CKIα isoforms is the presence or absence of two inserts, L and S. *From Pulgar et al. (1996).

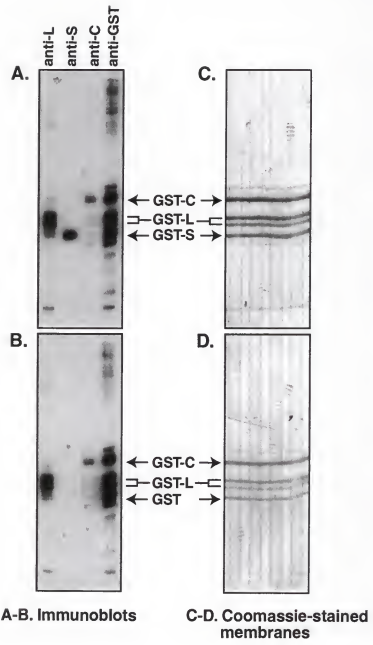


Figure 3-5. The affinity-purified CKI antibodies are specific for their respective immunogen sequences. Affinity-purified GST fusion proteins were run together on a SDS-Gel, transferred to a PVDF membrane, and probed with the CKI antibodies. Note that the GST-L preparation revealed a doublet, with the top band at the molecular weight position corresponding to the intact GST-L, and the lower band presumably corresponding to a degradation product. Note that each CKI antibody selectively recognizes its own immunogen.

specificities were tested on western blot containing all three immunogens: GST-L, GST-S and GST-C. Each antibody only recognized its corresponding immunogen (Fig. 3-5).

To determine the number and the identity of CKIα isoforms present in chicken brain, total chicken brain lysates were probed with the three antibodies. Only faint bands were produced and they were poorly resolved against a high background. Therefore, we analyzed a preparation enriched in total casein-binding proteins. The results, shown in Figure 3-6A, demonstrated the presence of five bands in the range of 37-42 kDa that were recognized by anti-C. Two of these five were also recognized by anti-S and two by anti-L, including the larger anti-S-positive band. These results are consistent with the identification of four of these five anti-C positive bands as CKIα, CKIαS, CKIαL and CKIαLS, in order of smallest to largest. The identity of the fifth band, just above CKIαLS, remains to be clarified. In addition, anti-C revealed a strong band at ~50 kDa, which was also recognized by anti-S and barely by anti-L, and a ~47 kDa band, which was also stained by the CKIε antibody (Fig. 3-6B). An antiserum prepared against a synthetic peptide consisting of the amino acids flanking, but excluding, the L insert (Pulgar et al., 1996) also revealed bands at the positions of CKIα and CKIαS, as well as some bands of larger size and one smaller. The latter may represent a degradation product. These results, taken together, provide evidence for the expression, at the protein level, of all four alpha isoforms in chicken brain.

Two different antibodies, anti-C and anti-S, both indicated that the two isoforms lacking the L insert (CKIα and CKIαS) are far more abundant in brain lysates than those containing this insert (CKIαL and CKIαLS). This is consistent with findings in rat (Zhang et al., 1996) and human (Kuret et al., 1997) brain. It is likely that this is a

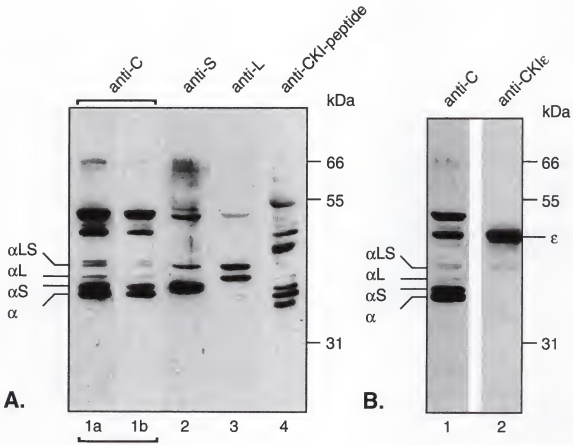


Figure 3-6. Immunoblot analysis of CKI isoforms in brain. **A:** Equal amounts of brain casein-binding proteins (see Methods) were applied to each of four lanes of an SDS gel and transferred to a PVDF membrane. The membrane was cut into four corresponding pieces which were incubated with anti-C (0.2μg/ml, lanes 1), anti-S (0.4μg/ml, lane 2), anti-L (1μg/ml, lane 3), and anti-CKI-peptide antiserum (1:5000, lane 4). Lane 1b is a shorter exposure of the Lane 1a. **B:** Brain casein-binding proteins were blotted with anti-C (0.2 μg/ml, lane 1), after which the membrane was stripped and reblotted with a monoclonal anti-CKIε (0.25μg/ml, lane 2).

reflection of relative levels of the mRNAs, rather than differences in protein stability, because the smaller two transcripts also predominated in chicken brain cDNA (Green and Bennett, 1998).

CKIε was first identified in human (Fish et al., 1995), and recently also in *Xenopus* (Peters et al., 1999). The appearance of an anti-C-positive band around 47 kDa position that was also strongly stained by a monoclonal antibody specific for mammalian CKIε suggests that this isoform is also expressed in chicken brain and has significant sequence homology to the mammalian CKIε. Assuming the expression of additional CKI isoforms also extends to chicken, other anti-C-positive bands may well represent CKIβ (39 kDa), CKIδ (49 kDa), or CKIγ (51 kDa). All of them have been detected in mammalian brain at the mRNA level (Graves et al., 1993; Rowles et al., 1991; Zhai et al., 1995) and, in the case of CKIδ, at the protein level as well (Kuret et al., 1997). We also cannot exclude the presence of additional CKI family members that do not react with anti-C.

In summary, this western blot revealed evidence for the presence in chicken brain of proteins corresponding to the four alternatively spliced CKIα mRNA transcripts we had previously identified (Green and Bennett, 1998), as well as CKIε and other putative members of the CKI family.

Enrichment of CKIα and CKIαS in Purified NFAK

After the detection of the presence of four CKI alpha isoforms at the protein level in chicken brain, purified NFAK samples in parallel with total brain casein-binding proteins were analyzed. Anti-C gave two major bands in purified NFAK at the positions of CKIα and CKIαS (Fig. 3-7A). The identification of the larger band as CKIαS was confirmed

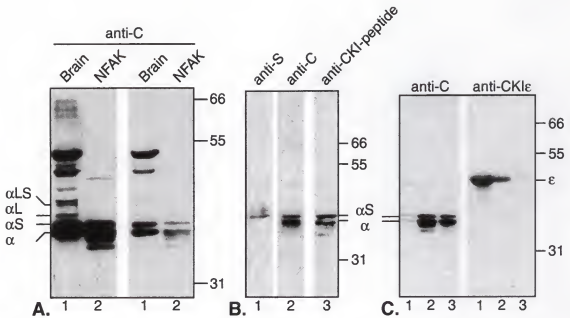


Figure 3-7. Immunoblot analysis of purified NFAK. **A:** Brain casein-binding proteins (lane 1) and casein-agarose purified NFAK (lane 2) were separated on an SDS gel, transferred, and blotted with anti-C (0.2 μg/ml). Shown are long (left) and short (right) chemiluminescent exposures. **B:** Casein-agarose purified NFAK was blotted with anti-S (0.4 μg/ml, lane 1), anti-C (0.2μg/ml, lane 2), and anti-CKI-peptide antiserum (1:4000, lane 3). **C:** NFAK fractions from progressive steps in the purification were blotted with anti-C (0.2 μg/ml) after which the membrane was stripped and reblotted with monoclonal anti-CKIε (0.25 μg/ml). Lane 1, high salt extract (75μg); Lane 2, Sephacryl-purified NFAK (4.5μg); Lane 3, casein-agarose purified NFAK (0.8μg).

with anti-S (Fig. 3-7B). No bands in position corresponding to CKIαL or CKIαLS or larger components were detected, although a smaller, presumptively proteolytic, fragment did appear. The anti-peptide antibody also detected only bands corresponding to CKIα and CKIαS. The composition of samples from progressive steps in the NFAK purification was also followed (Fig. 3-7C). It appears that CKIα and CKIαS were clearly enriched in the more purified NFAK samples compared with a crude fraction, whereas the CKIε signal was drastically diminished.

Immunodepletion of NFAK Activity

The fact that CKIα and CKIαS were the only immunoreactive components in purified NFAK is consistent with the in-gel kinase assay results, which indicated NFAK activity at the position of 36-40 kDa. However, it remained to be shown that these two isoforms account for all the NFAK activity. Thus, we conducted an immunoprecipitation experiment, in which proteinA-agarose beads were coated with different CKI antibodies and then incubated with purified NFAK. The beads with bound antigen-antibody complexes were sedimented, and the NFAK activity in both beads and supernatant fractions were quantitated with the in vitro kinase assay (Fig. 3-8). Uncoated beads (lane 3b) and beads coated with normal rabbit serum (lanes 4b and 5b) failed to bind any NFAK. Similarly, beads coated with anti-L antibody contained no NFAK activity (lanes 10b and 11b), and the activity remained in the supernatants (lane 10s and 11s). In contrast, beads coated with anti-peptide antiserum were able to bind 100% of the NFAK activity (lanes 6b and 7b), and beads coated with anti-S antibody were able to bind ~50% (lanes 8b and 9b). Doubling the amount of anti-S did not substantially increase the bound NFAK activity, which indicates that the amount of antibody used was saturating. The

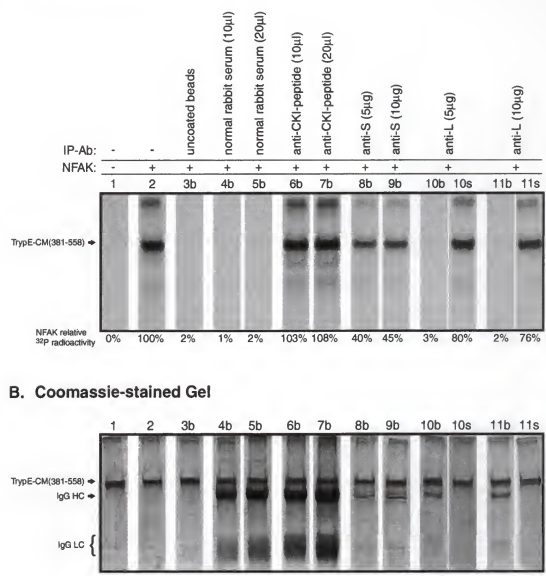


Figure 3-8. Immunoprecipitation of NFAK activity. Protein-A agarose beads were coated with appropriate antibodies or control immunoglobulins and then incubated with casein-agarose purified NFAK (see Methods). Equal aliquots of beads (b) and supernatants (s) were assayed for activity towards CM:381-558. Reaction mixtures were separated by SDS-PAGE and incorporation of 32P into the substrate was visualized by autoradiography (A) of the stained gel (B). Radioactivity was also quantitated by phosphorimager analysis and indicated below each lane relative to that obtained with the untreated kinase (Lane 2). CM:381-558 and the IgG heavy (HC) and light (LC) chains are indicated on the stained gel (B).

anti-C antibody, however, did not pull down a significant percentage of NFAK (data not shown). One possible explanation is that the anti-C antibody, which was raised against denatured GST-C, did not bind native CKI with sufficient affinity. In conclusion, the immunodepletion study demonstrated that CKIα and CKIαS together account for essentially all NFAK activity in purified NFAK.

Summary and Discussion

It was previously shown that the kinase activity bound to NFs (NFAK) has CKI-like properties and that it phosphorylates only those sites in NF-M that conform to a CKI recognition motif (Hollander et al., 1996). The present results show that, of all the CKI family members expressed in brain, only two, CKIα and CKIαS, were detectable in the purified kinase and that these two together were sufficient to account for the NFAK activity. None of the larger components recognized by anti-C remained in purified NFAK. Specifically, CKIε was progressively eliminated, and the two CKIα isoforms containing the L insert were also not detectable. If purified NFAK contains some other kinase(s) not reactive with our antibodies, this kinase does not contribute measurably to NFAK activity as defined by phosphorylation of the NFAK specific substrate, because no activity remained after immunoprecipitation by anti-CKI peptide. It is noteworthy that human CKIα1 and CKIα2 (corresponding to CKIα and CKIαS respectively) are tightly and selectively associated with paired helical filaments purified from Alzheimer's disease brain (Kuret et al., 1997) and may account for the activity of a paired helical filament-associated kinase (Jicha et al., 1999).

As detailed in chapter 1, CKIα has been detected in several subcellular locations, including plasma membrane (Brockman and Anderson, 1991), mitotic spindles (Brockman et al., 1992), synaptic vesicles (Gross et al., 1995) and nucleus (Gross et al., 1999), although these studies did not unambiguously discriminate among the four isoforms. Our present results suggest that two specific CKIα isoforms selectively associate with, and phosphorylate neurofilaments. The basis for such association remains to be determined. It is possible that CKIα and CKIαS directly bind to the cytoskeleton or associate with cytoskeleton-bound vesicular organelles. The exclusion of isoforms containing the L insert from purified NFAK may be explained by several possibilities, such as (1) the L insert interferes with direct binding to NFs; (2) the L insert mediates association with a structure or compartment that renders CKIαL and CKIαLS inaccessible to NFs; (3) the L containing isoforms are of relatively low abundance and/or are selectively susceptible to degradation. In chapter 4, evidence will be presented suggesting that the L insert is able to direct the nuclear targeting of CKIαL and CKIαLS, thus may help to sequester them away from NFs in the cytoplasm. Meanwhile the absence of other members of the CKI family in purified NFAK also raises questions as to their intracellular compartmentalization in neurons and their ability to bind to or phosphorylate NF proteins in vivo.

At present, the significance and regulation of NF tail domain phosphorylation are still poorly understood. It is clear that several different protein kinases are involved. The identification of two specific members of the CKI family in association with NFs sets the stage for further studies of the role and regulation of phosphorylation of CKI target sites in NF-M function (discussed in chapter 5).

CHAPTER 4
DIFFERENTIAL SUBCELLULAR LOCALIZATION OF FOUR CASEIN KINASE I ALPHA ISOFORMS

Introduction

In the previous chapter, two of the four CKI alpha isoforms (CKIα and CKIαS) were identified as a neurofilament-associated kinase (NFAK) that can selectively phosphorylate five specific serine residues on the chicken NF-M tail. It is tempting to speculate that the absence of the other two isoforms (CKIαL and CKIαLS) in the purified NFAK may be explained by the possibilities that the L insert either interferes with association to NFs or causes special compartmentalization to render CKIαL and CKIαLS inaccessible to NFs. In this chapter, we present evidence suggesting that the four isoforms are differentially localized in several non-neuronal cell types, and particularly the two isoforms containing the L insert are targeted to the nucleus and associate with nuclear structures.

Isoform-Specific Compartmentalization of Protein Kinase and Phosphatase

Isoforms generated by gene duplication and/or alternative splicing display biological diversity not only because they can exhibit related but different functional properties but also because they can be segregated into different intracellular sites within the same cell. Sorting has been observed in a wide range of molecules, including structural molecules, receptors, ion channels, enzymes, and signaling molecules (Gunning et al., 1998).

One of the earliest and also probably the most comprehensive analysis of differential sorting of multiple isoforms of a kinase comes from studies of the protein kinase C (PKC) family. Each of the eight different PKC isoforms were overexpressed in NIH-3T3 cells (Goodnight et al., 1995). Prior to activation, the majority of the isoforms are diffusely distributed through the cytoplasm, whereas upon activation, each activated PKC isoform specifically associates with a particular intracellular structure, including nuclear membranes, Golgi, endoplasmic reticulum, actin microfilaments, and plasma membrane. This finding suggests that the PKC substrate specificity may be mediated by the isoform-specific compartmentalization that restricts each PKC isozyme to the location of its target protein. A variety of other kinases also demonstrate isoform specific localization. For instance, the S6 kinase isoform p85 has a predominantly nuclear localization, whereas the isoform p70 is primarily cytoplasmic (Reinhard et al., 1994). Similarly, the nucleus versus cytoplasm sorting is also observed for α-, β-, and δ-isoforms of calmodulin-dependent protein kinase (CaM kinase) (Brocke et al., 1995; Srinivasan et al., 1994). The MAP kinase MEK5 isoforms are also differentially sorted in the cytoplasm, with the α isoform associating with the actin cytoskeleton and β isoform being primarily cytosolic (English et al., 1995). Recently, accumulating evidence has mounted indicating that the type II cAMP-dependent protein kinase (PKA) is targeted to many different cellular structures by association with A-kinase anchoring proteins (AKAPs) (Scott, 1997). Isoform-specific sorting also exists in the case of protein phosphatases. For example, two isoforms of calcineurin, the catalytic subunit of a calmodulin-dependent protein phosphatase, are sorted between the nucleus and cytosol (Usuda et al., 1996). The protein

phosphatase type IC α and δ isoforms also differ in their sorting between the nucleus and cytoplasm and within the cytoplasm (Murata et al., 1997).

For protein kinases or phosphatases with multiple subunits, sorting usually occurs by association of the regulatory subunit with targeting proteins or by association of the catalytic subunit with the targeting subunit. For instance, PKA is targeted to different intracellular compartments by association of its regulatory subunit with as many as 36 unique AKAPs (Scott, 1997). In the case of protein phosphatase 1, it is targeted to its substrates by virtue of a targeting subunit that associates with its catalytic subunit (Hubbard and Cohen, 1993). As for CaM kinase, a nuclear localization signal (NLS) has been identified in each of the three nuclear isoforms. More interestingly, nuclear isoforms and cytoplasmic isoforms of CaM kinase can form a heteromultimer, and the ratio of nuclear to cytoplasmic subunits was shown to determine the subcellular targeting of CaM kinase between the nucleus and the cytoplasm (Srinivasan et al., 1994).

CKI, however, is a monomeric kinase. Although it has been identified in different intracellular compartments, neither targeting sequence nor anchoring protein for specific localization has been reported, except that a prenylation motif present exclusively in several yeast CKI homologues is found to mediate association with the plasma membrane (see chapter 1). In the following sections, data will be presented to demonstrate that a nuclear targeting sequence is responsible for the differential localization of the four CKI alpha isoforms between the nucleus and the cytoplasm.

Differential Compartmentalization of CKI Isoforms

One prominent feature of the CKI family is its widespread distribution. CKI has been shown to localize in the cytosol and nucleus, associate with vesicular structures,

cytoskeleton and plasma membrane. This broad distribution pattern of CKI may be explained by the fact that there are many different CKI isoforms and each one of them may be differentially localized. As discussed in chapter 1, our current knowledge about the subcellular localization of CKI is largely based on the detection of the CKI-like activity through biochemical fractionation studies, or the immunoreactivity of some CKI antibodies that are not isoform-specific. Therefore, still very little is known about the specific localization and regulation of each individual CKI isoform. Among all the known vertebrate CKI isoforms, CKIα has been better characterized in terms of its intracellular distribution. Several reports have appeared suggesting the association of CKIα with mitotic spindles (Brockman et al., 1992), with synaptic vesicles (Gross et al., 1995) and with nuclear structures (Gross et al., 1999). However, none of these studies was able to discriminate among the different α isoforms. Thus, we carried out further studies to determine the specific localization of each of the four CKIα isoforms.

Experimental Results

The Four CKI Alpha Isoforms are Differentially Compartmentalized

By using the PSORT world wide web server (http://psort.nibb.ac.jp/) to analyze and predict protein sorting signals coded in the amino acid sequence of the entire CKI alpha sequence, one putative nuclear localization signal (NLS) (PVGKRKR), located in the L insert, was identified (Fig 4-1). Currently, NLS are classified into three categories, two of which have been well studied: the classical type of NLS (as typified by SV40 large T antigen) and the bipartite NLS (as typified by nucleoplasmin) (Dingwall and Laskey,

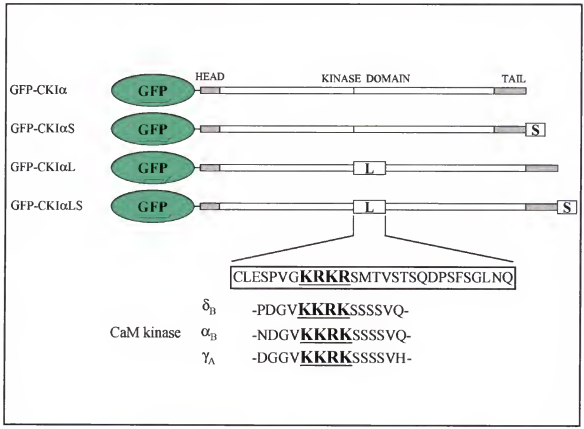


Figure 4-1. Diagram of four GFP-CKI alpha fusion constructs and a putative NLS in the L insert. The L insert contains a putative nuclear localization signal PVGKRKR, and a similar sequence is also present among three nuclear isoforms of Calmodulin-dependent protein kinase (CaM).

1991; Hicks and Raikhel, 1995). The putative NLS identified in CKI alpha matches the classical type of NLS [$(K/R)_4$; $(K/R)_3$ H/P; $PX_{1-3}(K/R)_3$] (Hicks et al., 1995). A similar sequence (PDGVKKRK) has been confirmed to be an NLS in the δ_B isoform of Ca^{2+}/ Calmodulin-dependent protein kinase (CaM) (Srinivasan et al., 1994) (Fig 4-1). Therefore, we speculated that the L insert might direct selective nuclear targeting. In contrast, none of the known sorting signals was identified in the S insert. Thus, we also hypothesized that the S insert may not be involved in the regulation of intracellular compartmentalization.

Because the only difference in sequence between the four CKI alpha isoforms is the presence or absence of the L and the S inserts, we set out to test the above hypotheses by analyzing and comparing the subcellular localization of the four CKI alpha isoforms. In doing so, the CKI alpha sequence was engineered behind the C-terminus of an enhanced GFP tag (Fig. 4-1), and the chimeric construct was transfected into COS-7 and NIH-3T3 cells. The transfection efficiency for both cell lines was low, with about 1-5% for COS-7 cells and <1% for NIH-3T3 cells. However, a consistent distribution pattern for each construct emerged from the transfected cells. In both COS-7 (Fig. 4-2) and NIH-3T3 (Fig. 4-3) cells, the two isoforms with the L insert were concentrated in the nucleus, whereas the two isoforms without the L insert were concentrated in the cytoplasm. This observation confirms the prediction that the L insert is able to target CKIαL and CKIαLS to the nucleus. It was also noted that the cytoplasmic staining of GFP-CKIα and GFP-CKIαS always exhibited two patterns in COS-7 (Fig. 4-2 A and B), punctate and diffuse. The ratio between these two subtypes varied in different experiments, but they together

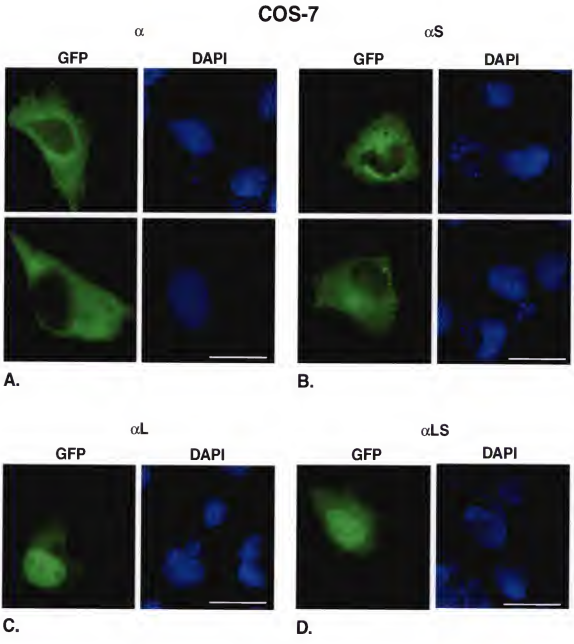


Figure 4-2. Differential localization of four GFP-CKI alpha constructs in COS-7 cells. Cells were transiently transfected with four GFP-CKI alpha constructs (see Methods). Two major patterns of GFP signals were observed in both GFP-CKIα (A) and GFP-CKIαS (B) transfected COS-7 cells: punctate (top) and diffuse (bottom) (detailed in text). Note both GFP-CKIα and GFP-CKIαS proteins (A-B) were concentrated in the cytoplasm, whereas GFP-CKIαL and GFP-CKIαLS proteins (C-D) were concentrated in the nucleus. Bar=10 μm.

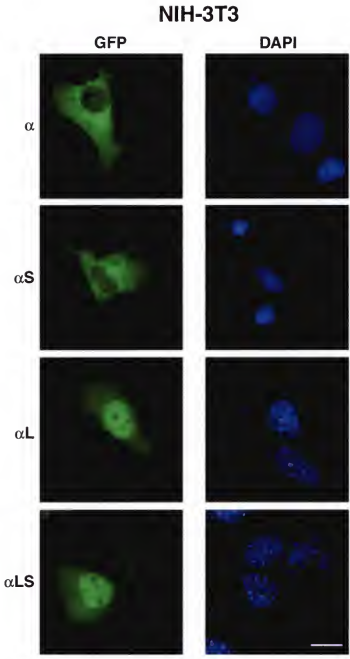


Figure 4-3. Differential localization of four GFP-CKI alpha constructs in NIH-3T3 cells. Cells were transiently transfected with four GFP-CKI alpha constructs (see detail in Methods). Note, similar to COS-7 cells (Fig. 4-2), both GFP-CKIαL and GFP-CKIαLS proteins were concentrated in the nucleus, whereas GFP-CKIα and GFP-CKIαS proteins were concentrated in the cytoplasm. Bar=10 μm.

accounted for 80-90% of the total population of transfected cells. The other 10-20% population of transfected cells includes either cells (10-15%) exhibiting very bright GFP signals concentrated in a perinuclear blob region and abnormal cellular morphology, or cells (~5%) showing almost equal intensity of GFP signals in both the cytoplasm and the nucleus (data not shown). Speculations about the underlying mechanism for these expression patterns will be addressed in the discussion.

The Two Nuclear CKI Alpha Isoforms Co-localize with Nuclear Speckles

Although GFP-CKIαL and GFP-CKIαLS in transfected COS-7 and NIH3T3 cells exhibited diffuse signals (Fig. 4-2 and Fig. 4-3), it may simply reflect the fact that the distinctive subnuclear structures were obscured by intense diffuse signals due to the over-expressed proteins. It has been previously documented that pre-extraction can result in the loss of soluble cytoplasmic and nuclear components, thus helping to reveal the extraction-resistant signals which associate with distinct nuclear structures (Bisotto et al., 1995; Boronenkov et al., 1998; Fey et al., 1986; Vyakarnam et al., 1998). Therefore, in order to know if GFP-CKIαL and GFP-CKIαLS could possibly associate with some nuclear structures, we briefly pre-extracted transfected cells with TX-100 before fixation. Pre-extracted COS-7 cells revealed many bright granules compared with untreated COS-7 cells (Fig. 4-4). This was also true for NIH-3T3 cells (data not shown).

The granular pattern of GFP-CKIαL and GFP-CKIαLS in the nucleus was reminiscent of the morphological appearance of nuclear speckles that contain snRNP and non-snRNP splicing factors (Fu and Maniatis, 1990; Spector et al., 1991; Spector et al., 1983). In order to determine if GFP-CKIαL and GFP-CKIαLS might be associated with nuclear speckles, a typical nuclear speckle marker, the Y12 monoclonal anti-Sm antibody

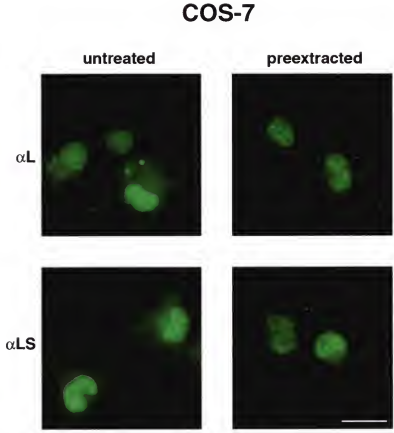


Figure 4-4. Nuclear-targeted GFP-CKIαL and GFP-CKIαLS exhibit both diffuse and granular signals. After transient transfection, COS-7 cells were either directly fixed (left panel) or briefly pre-extracted (right panel) with 0.2% Triton X-100 and then fixed for fluorescence analysis (see Methods). Note that, with the removal of Triton X-100 extractable signals from both the nucleus and the cytoplasm, many discrete speckles were revealed in the nucleus. Bar=10 μm.

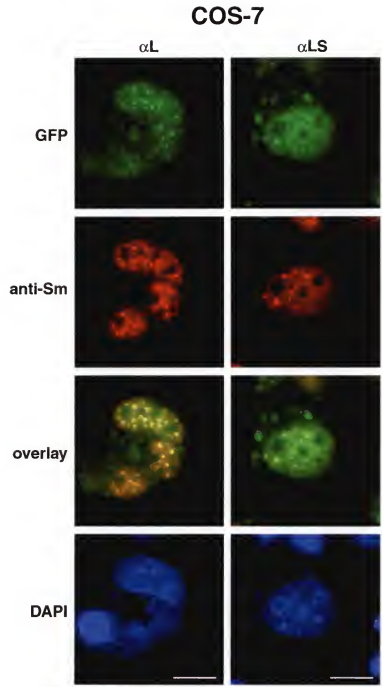


Figure 4-5. GFP-CKIαL and GFP-CKIαLS co-localize with nuclear speckles in COS-7 cells. COS-7 cells were transiently transfected with either GFP-CKIαL (left panel) or GFP-CKIαLS (right panel). After pre-extraction and fixation, cells were incubated with Y-12 monoclonal anti-Sm antibody for staining of snRNPs at nuclear speckles. The co-localization between GFP signals (green) and anti-Sm staining (red) is clearly demonstrated in the overlay (yellow). Bar=10μm.

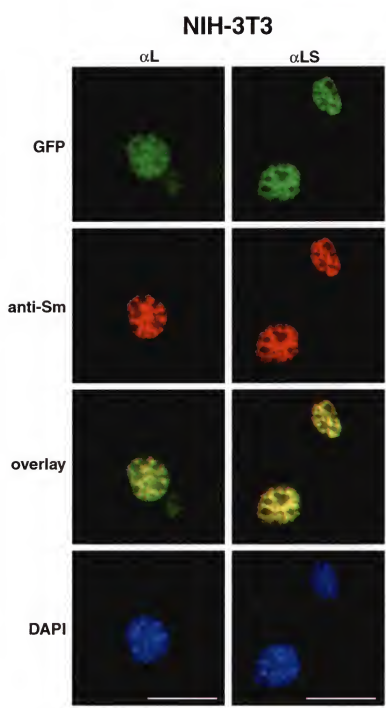


Figure 4-6. GFP-CKIαL and GFP-CKIαLS co-localize with nuclear speckles in NIH-3T3 cells. NIH-3T3 cells were transiently transfected with either GFP-CKIαL (left panel) or GFP-CKIαLS (right panel). After pre-extraction and fixation, cells were incubated with Y-12 monoclonal anti-Sm antibody for staining of snRNPs at nuclear speckles. The co-localization between GFP signals (green) and anti-Sm staining (red) is clearly demonstrated in the overlay (yellow). Bar=10 μm.

(Y12 mAb), was used to stain splicesome snRNPs (Moussa et al., 1994; Puvion-Dutilleul et al., 1994). Granular fluorescence signals of GFP-CKIαL and GFP-CKIαLS in both COS-7 (Fig. 4-5) and NIH-3T3 (Fig. 4-6) cells co-localized completely with anti-Sm staining, suggesting association of CKIαL and CKIαLS with nuclear speckles.

Nuclear Co-localization is Sensitive to Transcription Inhibition.

It has been shown that nuclear speckles are highly dynamic structures that respond specifically to activation of nearby genes, and this dynamic event is dependent upon RNA polymerase II-mediated transcription (Misteli and Spector, 1996). One important feature about the dynamics of nuclear speckles is that they appear to be larger and rounder when transcription is inhibited (Carmo-Fonseca et al., 1991; Spector et al., 1983).

To see if the localization of GFP-CKIαL and GFP-CKIαLS are sensitive to transcription, transfected COS-7 cells were treated with amanitin, a specific inhibitor of RNA polymerase II. Shown in Fig. 4-7, the speckles of both GFP fusion proteins and anti-Sm became larger and rounder, and more importantly the co-localization between them still occurred. Amanitin, however, did not affect the distribution patterns of GFP fusion and anti-Sm in NIH-3T3 cells to a significant extent (data not shown). The phenomenon observed in COS-7 cells is in agreement with the observation that endogenous CKIα (not known which isoform), concomitantly with snRNPs, reorganized to larger and brighter nuclear structures when RNP-II activity was inhibited (Gross et al., 1999). The finding that the nuclear isoforms of CKIα relocate identically with snRNPs when RNP-II-mediated transcription is inhibited implies that CKIαL and CKIαLS

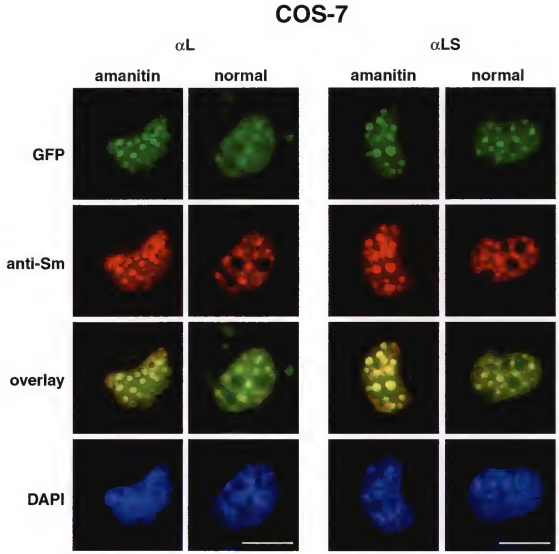


Figure 4-7. The co-localization of GFP-CKIαL and GFP-CKIαLS with nuclear speckles persists upon inhibition of transcription. Post-transfected COS-7 cells were incubated with amanitin, a specific RNA polymerase II inhibitor, and then pre-extracted, fixed and followed by the incubation with Y-12 monoclonal anti-Sm antibody. Shown here, the GFP-CKIαL and GFP-CKIαLS granules, concomitantly with nuclear speckles, appeared larger and rounder after amanitin treatment. Also, shown in the overlay, the co-localization of GFP-CKIαL and GFP-CKIαLS with nuclear speckles still occurred. Bar=10μm.

associate, either directly or indirectly, with snRNP splicing factors, and that this association is not dependent on transcription.

The Two Cytoplasmic CKI Alpha Isoforms are not Restricted to either the Golgi Complex or the Endoplasmic Reticulum Structure.

Previously, it was demonstrated that immunofluorescence staining of an antibody raised to α-CKI is partially coincident with both Golgi and endoplasmic reticulum markers in the cytoplasm (Gross et al., 1995). However, due to the lack of selectivity, this α-CKI antibody could not discriminate between the four CKIα isoforms (see chapter 1). In Fig. 4-2, we demonstrated that the four CKI alpha isoforms are differentially localized, with the CKIαL and CKIαLS being predominantly nuclear, and the CKIα and CKIαS being primarily cytoplasmic. Although both the GFP-CKIα and GFP-CKIαS signals appeared almost everywhere in the cytoplasm, it was of interest to ask whether there might be some significant association of these two isoforms with either the Golgi or the endoplasmic reticulum structure.

To address this question, we transfected the GFP-CKIα and GFP-CKIαS constructs into COS-7, NIH-3T3 and LLC-PK cells, which were then stained with wheat germ agglutinin (WGA), a marker for the Golgi complex, and concanavalin A (ConA), a marker for the endoplasmic reticulum. As shown for all three cell lines (Fig. 4-8, 4-9, 4-10), WGA stained the Golgi apparatus as a juxtanuclear reticular structure, whereas ConA labeled the endoplastic reticulum as a wide perinuclear region, and these observations are consistent with those previously documented (Virtanen et al., 1980). Furthermore, it is shown for all three cell lines that both GFP-CKIα and GFP-CKIαS signals did not seem to significantly overlap with either the granular staining of the Golgi marker or the vesicular staining of the ER marker. However, given that there were some

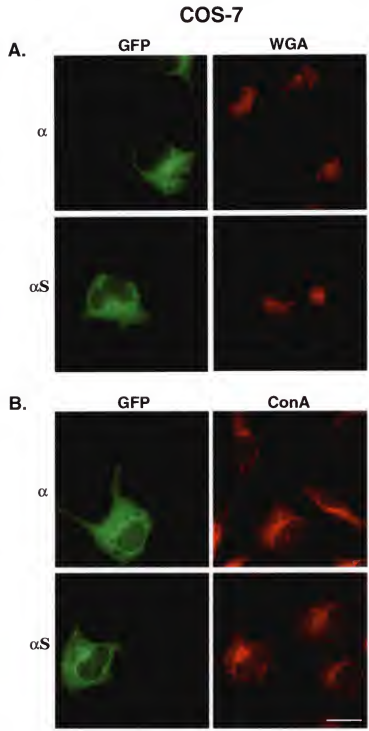


Figure 4-8. Comparison of the distribution patterns of GFP-CKIα and GFP-CKIαS with two endomembrane structures in COS-7 cells. Both GFP-CKIα and GFP-CKIαS transfected cells were probed with either WGA (Golgi marker) (A) or ConA (ER marker) (B) (see detail in Methods). Bar=10 μm.

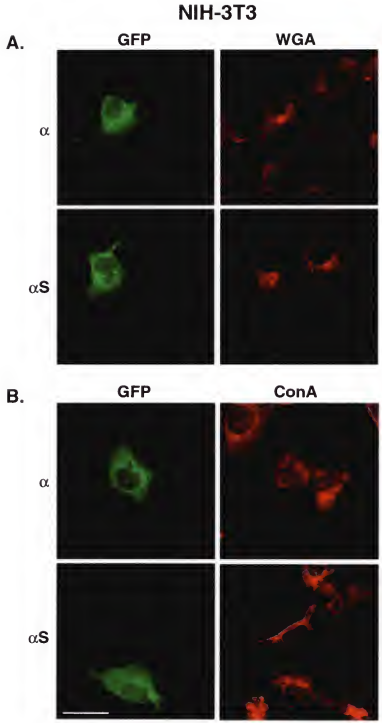


Figure 4-9. Comparison of the distribution patterns of GFP-CKIα and GFP-CKIαS with two endomembrane structures in NIH-3T3 cells. Both GFP-CKIα and GFP-CKIαS transfected cells were probed with either WGA (Golgi marker) (A) or ConA (ER marker) (B) (see detail in Methods). Bar=20μm.

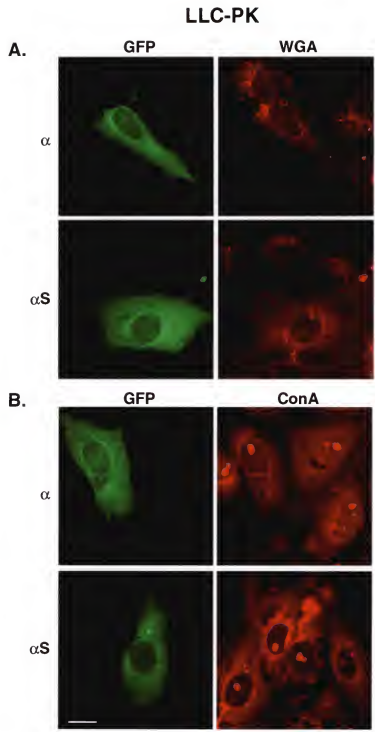


Figure 4-10. Comparison of the distribution patterns of GFP-CKIα and GFP-CKIαS with two endomembrane structures in LLC-PK cells. Both GFP-CKIα and GFP-CKIαS transfected cells were probed with either WGA (Golgi marker) (A) or ConA (ER marker) (B) (see detail in Methods). Bar=10μm.

GFP-CKIα and GFP-CKIαS signals present in the Golgi and more notably in the endoplasmic reticulum regions, further investigation is required to determine whether or not this observation reflects the association of a certain portion of CKIα and CKIαS isoforms with these two endomembrane structures. It must be taken into consideration that transiently transfected cells tend to overexpress the corresponding protein, thus some of the intracellular localization pattern may not be a true reflection of the endogenous subcellular distribution. For instance, it is possible that overexpressed proteins may saturate the binding sites to a particular intracellular structure so that a significant portion of the exogenous proteins may randomly distribute in the cell. Currently, the immunofluorescence studies by use of some isoform-specific antibodies are underway to further dissect the subcellular distribution of the two alpha isoforms in the cytoplasm.

The S Insert may not be Involved in the Regulation of the Intracellular Distribution of the Four CK1 Alpha Isoforms.

It should be noted that the GFP-CKIα and GFP-CKIαS demonstrated very similar intracellular distribution patterns in the cytoplasm of all three cell lines examined (Fig. 4-8, 4-9, 4-10). Also, both GFP-CKIαL and GFP-CKIαLS showed almost identical patterns of nuclear speckle co-localization (Fig. 4-5, 4-6). Taken together, it is suggested that the S insert may not be involved in the determination of the intracellular localization of the four CKIα isoforms. The potential functions of the S insert will be addressed in the discussion.

However, because the S insert is a very short sequence and located at the very C-terminal end of the GFP fusion protein, it is possible that the S insert may be degraded due to proteolysis, and thus the difference, if there is any, between isoforms with and without the S insert can not be detected. Therefore we tested whether or not the S insert is

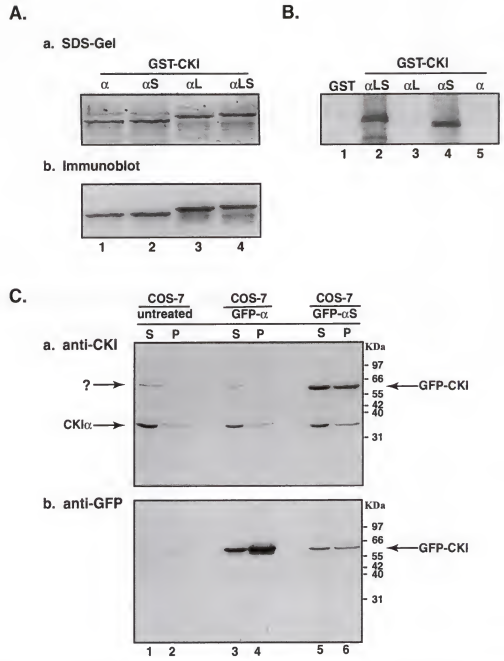


Figure 4-11. GFP-CKIαS proteins expressed in COS-7 cells still retain the S insert at the C-terminus. **A.** GST-CKI fusion proteins (prepared as detailed in Methods) were run on an SDS-Gel (a), and then transferred to a PVDF membrane (b) that was probed with affinity-purified anti-C antibody (see chapter 3). **B.** The GST-CKI samples used in A. were probed with an anti-peptide CKI antibody (StressGen), and shown here is the immunoblot. Note that this CKI antibody only recognizes the recombinant CKI proteins with the S insert. **C.** Cell lysates from untreated or transfected COS-7 cells were prepared in the presence of 0.2% Triton X-100 (detailed in Methods). After centrifugation, both the soluble (S) and the pellet (P) fractions were probed with either the anti-peptide CKI antibody (a) or a monoclonal anti-GFP antibody (b).

still maintained intact after the expression of the GFP-CKIαS by using an anti-peptide CKI antibody, which is raised against a sequence of the CKIαS C-terminus containing the entire S insert. Based on the fact that almost the entire immunogene sequence of this antibody is the S insert, we speculated that this antibody may selectively recognize the S-containing isoforms. To analyze the specificity of this antibody among different CKI alpha isoforms, recombinant GST fusion proteins (Fig. 4-11-A) corresponding to all four full-length CKI alpha isoforms were probed on western blots. Among the four GST-CKI fusion proteins, only the two isoforms containing the S insert were recognized by the anti-peptide CKI antibody (Fig. 4-11-B), indicating that this CKI antibody is specific for the S-containing isoforms.

Subsequently, the CKI antibody, together with a monoclonal GFP antibody, was utilized to probe cell lysates from either untreated or GFP-CKIα / GFP-CKIαS transfected COS-7 cells. Shown in Fig. 4-11-C, two bands appeared in the supernatant of untreated COS-7 cell lysate, with the bottom band (~37 kDa) being much more abundant than the top band (~60 kDa) (a, lane 1). In the pellet fraction of untreated COS-7 cell lysate (a, lane 2), however, only the ~37 kDa band was weakly stained, presumably because the levels of both proteins were proportionally reduced in the pellet fraction so that the ~60 kDa band was hardly detectable. The two-band pattern revealed by this CKI antibody in COS-7 cells is very similar to that presented for the same antibody in rat brain (Stressgene Corp). According to the manufacturer, the ~37 kDa band corresponds to α isoform, while the identity of the ~60 kDa band is claimed to correspond to CKIε.

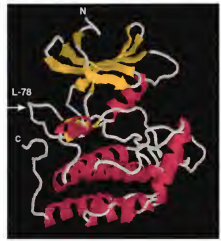
Meanwhile, the anti-GFP antibody revealed one band that corresponds to the GFP fusion protein and was closely positioned, but not overlapping, with the endogenous ~60

kDa band in both the supernatant and the pellet fraction of either GFP-CKIα (b, lane 3-4) or GFP-CKIαS (b, lane 5-6) transfected COS-7 cells. In contrast, the CKI antibody only recognized the GST fusion protein band in GFP-CKIαS transfected COS-7 cells. Taken together, it is confirmed that the S insert is still retained in overexpressed GFP-CKIαS fusion proteins. This result eliminates the possibility that the absence of the S insert in GFP-CKI fusion proteins due to the proteolytic degradation might account for the lack of any significant difference in subcellular localization patterns between the CKI alpha isoforms with and without the S insert.

Summary and Discussion

This is the first time that a specific sequence of CKI is demonstrated to be responsible for nuclear targeting. Since there exists only one consensus NLS sequence in the L insert, the likelihood of the four basic residues KRKR being responsible for the nuclear localization is hardly questionable. Besides, a similar sequence has already been identified as NLS in nuclear isoforms of CaM kinase. Based on the crystal structures of yeast *cki*1 (Xu et al., 1995) and truncated CK1δ (Longenecker et al., 1996), and highly conserved primary sequences of CKI isoforms, the L insert is positioned on Loop-78, which is shown in the ribbon diagram of yeast Cki molecular structure obtained from RasMol (Sayle and Milner-White, 1995) (Fig. 4-12). Due to the obvious flexibility and accessibility of Loop-78, the L insert stands at a very good position for any possible interactions with, for instance, nuclear import apparatus.

A.



B.

putative NLS

1 CLESPVGKRKRSMTVSTSQDPSFSGLNQ 28

(12, 14, 16, 17, 18, 22)

Potential phosphorylation sites for protein kinase CKI:

S-16 S*MTVSTSQ

S-22 S*QDPSFSG

Potential phosphorylation site for protein kinase CKII:

T-17 TVSTSQD

Potential phosphorylation sites for protein kinase GSK3:

S-12 RKRSMTVS*

S-18 VSTSQDPS*

Potential phosphorylation site for protein kinase CaMII:

S-12 RKRSMTV

Potential phosphorylation site for protein kinase p70s6k:

T-14 RKRSMTVST

Potential phosphorylation site for protein kinase PKA:

S-12 RKRSMTV

Potential phosphorylation site for protein kinase PKG:

S-12 KRKRSMTV

Figure 4-12. Predicted features of the L insert primary sequence and location in the 3-D structure. **A.** The ribbon diagram of the molecular structure of yeast Cki. This diagram is obtained from RasMol (Sayle and Milner-White, 1995). The yeast Cki catalytic domain folds into two lobes, one dominated by β sheets (yellow), the other by α helices (pink). Based on the significant sequence homology between yeast and vertebrate CKI isoforms, the L insert is located in Loop-78 (arrow). **B.** The amino acid sequence of the L insert. Note that several potential phosphorylation sits are located adjacent to the NLS motif (box). * must be prior phosphorylated.

It is also worth noting that the L insert contains several potential phosphorylation sites (Fig. 4-12). Phosphorylation has been known to be able to inactivate NLS thus preventing nuclear import (Jans and Hubner, 1996). For example, it has been documented that nuclear transport of lamin B2 was inhibited by phosphorylation at two PKC sites adjacent to the NLS (Hennekes et al., 1993). Recently, it was shown that phosphorylation of NF-AT4 transcription factor by CKIα masked the nuclear import signal, thus inhibiting NF-AT4 nuclear translocation (Zhu et al., 1998). So, it would be of great interest to investigate if phosphorylation of these sites around PVGKRKR sequence can regulate the nuclear import of CKIαL and CKIαLS.

Previously, a near-consensus putative NLS (KKQKY) located in the catalytic domain of yeast CKI isoforms was suggested (Hoekstra et al., 1991). This sequence motif is well conserved in all CKI isoforms and was predicted to cause selective nuclear targeting (Gross and Anderson, 1998). However, our data here suggest that it is not competent to direct nuclear-targeting because GFP-CKIα and GFP-CKIαS that contain this putative motif were predominantly distributed in the cytoplasm. However, we cannot exclude the possibility that this sequence may not be well exposed for recognition in the 3-D structure of GFP-CKI fusion protein, or it may be inactivated by, for instance, phosphorylation.

It has been proposed that nuclear import and nuclear speckle targeting are two consecutive but distinct steps that may be mediated by different signals (Hedley et al., 1995). Previously, an arginine/serine-rich sequence has been suggested to be sufficient for subnuclear localization to speckle domains (Hedley et al., 1995). However, no such RS dipeptide motif has been identified in the CKI alpha sequence, thus further study is

required to elucidate the sequence motif that is either directly or indirectly responsible for the nuclear speckle targeting of CKIαL and CKIαLS.

It has been known for almost two decades that RNA polymerase II (RNP-II) can be phosphorylated by the CKI activity in vitro (Dahmus, 1981). But it is not known if this is true in vivo, and what specific isoforms of CKI could possibly be responsible. Here, we reported that the two L-containing CKI alpha isoforms were targeted to the nucleus and associated with nuclear speckles. Our in vitro observation is consistent with the in vivo observation that endogenous CKIα (not known which isoform) colocalized with nuclear speckles. The in vitro and in vivo evidence, taken together, clearly position these two specific isoforms of CKI alpha as potential candidates for phosphorylating RNP-II in vivo. Recently, it has been shown that at least four serine-arginine (SR)-rich splicing factors can be phosphorylated within the SR domain by recombinant CKIα activity in vitro (Gross et al., 1999). It is known that RNA splicing of pre-mRNA in eukaryotes occurs cotranscriptionally. Recent findings also suggest that hyperphosphorylated RNP-II interacts via its carboxyl-terminal domain (CTD) with SR-rich splicing factors, which provides further indication that RNP-II mediated transcription is coupled with pre-mRNA processing (Bentley, 1999). Given that nuclear speckles are known to be storage sites for both RNP-II and splicing factors, it is conceivable that the two L-containing CKI alpha isoforms may play an important role in the coordination of RNA transcription and splicing by phosphorylating both RNP-II and splicing factors.

As indicated in the result section, as many as four fluorescence patterns for either GFP-CKIα or GFP-CKIαS transfected COS-7 cells were observed, although only the two patterns representing the majority of transfected cells were shown in Fig. 4-2. 10-15% of

transfected cells (not shown) exhibited extraordinarily bright green fluorescence signals, particularly in a perinuclear blob region. This phenomenon is very similar to the description of a recently-characterized perinuclear structure termed as aggresome, which was generated by the overexpression of a cytosolic GFP fusion protein, and has been proposed to represent a general cellular response of misfolded proteins (Garcia-Mata et al., 1999). Since the perinuclear aggresome has been shown to interfere with correct Golgi localization and disrupt the normal distribution of microtubules, this population of transfected cells was not taken into further analysis for subcellular distribution of CKI alpha isoforms. The other population of transfected cells (5-10%), also not shown, exhibited almost equal intensity of GFP signals in both the cytoplasm and nucleus, indicating that a significant percentage of expressed GFP-CKIα or GFP-CKIαS proteins were also localized in the nucleus of these cells. Since the size of GFP-CKI fusion proteins is about 60 kDa which is below the minimal size requirement for selective nuclear import, it is likely that the GFP-CKIα and GFP-CKIαS signals could passively diffuse between the nucleus and the cytoplasm. As far as the 80-90% of either GFP-CKIα or GFP-CKIαS transfected cells are concerned, they exhibited either punctate or diffuse pattern of GFP signals (Fig. 4-2). Both types of cells did not look as bright as cells that form aggresome, indicating they may have a lower level of overexpression. Since cells with punctate signals usually looked brighter than cells with diffuse signals, it is possible that the granules in the punctate pattern might represent small aggregates of overexpressed proteins that are going to be transported for degradation. It should be noted that the majority of transfected NIH-3T3 and LLC-PK cells displayed only diffuse green fluorescence signals.

The co-localization studies suggested both CKIα and CKIαS did not significantly coincide with the Golgi apparatus, which is consistent with a previous report that almost no detectable CKI activity was found in the rat liver Golgi subcellular fraction (Lasa et al., 1997). More interestingly, that report also identified a third class of casein kinases, which is distinct from CK1 and CK2 based upon several biochemical properties, and termed G-CK because it can be specifically located to the Golgi apparatus. Since the G-CK has not been cloned yet, the sequence of this novel casein kinase member is not known. Also in that report, it was indicated that the majority of the CKI activity in the post-nuclear cellular fraction from rat liver is located in the endoplasmic reticulum and mitochondria fraction. Our results indicated that although the two CKI alpha isoforms were not restricted to the endoplasmic reticulum structure, they seemed to partially overlap with the endoplasmic reticulum at the perinuclear region. A subcellular fractionation study of transfected COS-7 cells would be helpful in determining if there is partial co-incidence with the endoplasmic reticulum structure, and if so, to what extent it would be.

Our results from three different cell lines together suggested that the S insert may not be involved in the regulation of subcellular localization. However, since transiently transfected cells tend to be saturated by overexpressed proteins, it is possible that any subtle difference in subcellular distribution between the isoforms with and without the S insert might be masked. Hopefully, localization of endogenous CKI alpha isoforms with isoform-specific antibodies may shed new light on this issue. As mentioned in chapter 1, the only identified α isoform in *Xenopus*, which contains the S insert, has been shown to have the capability of weakly phosphorylating tyrosine residues (Pulgar et al., 1996).

However, it is not clear yet as to whether or not the S insert is involved in the catalytic activity for tyrosine phosphorylation. Since both GST-CKIα and GST-CKIαS constructs have been cloned and expressed, a comparison between them in terms of the ability to phosphorylate tyrosine residues will help to clarify the possible catalytic function of the S insert.

In general, the regulatory mechanism of CKI alpha is still poorly understood. The study of rat CKIα and CKIαL revealed that the L insert may play a role in regulating the kinase catalytic activity (Zhang et al., 1996). In this study, we suggested that the L insert was able to specifically target the two L-containing CKI alpha isoforms to the nucleus. Compartmentalization has been recognized as a very important means of sequestering certain kinases to specific locations for more direct and efficient contact with their substrates. Previously, we have identified CKIα and CKIαS to be able to selectively associate with chicken neurofilaments and phosphorylate NF-M tail at five specific serine residues (Fu et al., 1999). Here, we presented evidence suggesting that the L insert mediates association of two isoforms containing the L insert with nuclear structures in non-neuronal cells, suggesting that the nuclear compartmentalization may render them inaccessible to NFs in neuronal cells. Taken together, it seems that alternative splicing of CKI alpha gene introduces an inserted sequence that can help sorting the four CKI alpha isoforms to different locations and thus perform specific functions.

CHAPTER 5
SUMMARY AND PERSPECTIVES

Summary

Casein Kinase I is a large protein kinase family, consisting of multiple distinct isoforms. As detailed in chapter 1, many in vitro substrates for CKI have been identified, but very few of them have been proven to be physiologically relevant targets in vivo, and very little is known about the specific CKI isoform that is responsible for the phosphorylation of a given substrate. In addition, although CKI has been detected in several different intracellular compartments, the isoform-specific distribution is largely unknown. To date, the biological picture of this kinase family is still not clear, and probably the most important reason being that there is a significant lack of understanding with regard to the differences in functions and regulation between different isoforms.

My studies during the past four years have been focused on the characterization of four alternatively spliced CKI alpha isoforms. In chapter 3, I presented biochemical and immunological evidence which together indicate that two CKI alpha isoforms (CKIα and CKIαS) are responsible for phosphorylating the chicken NF-M tail domain at five specific serine residues. This finding sets the stage for the study of potential effects of those CKI phosphorylation sites on NF morphology and NF-associated behaviors. In chapter 4, I addressed another very important issue about the CKI alpha isoforms, which is, the differential localization of the four isoforms, in particular, the specific

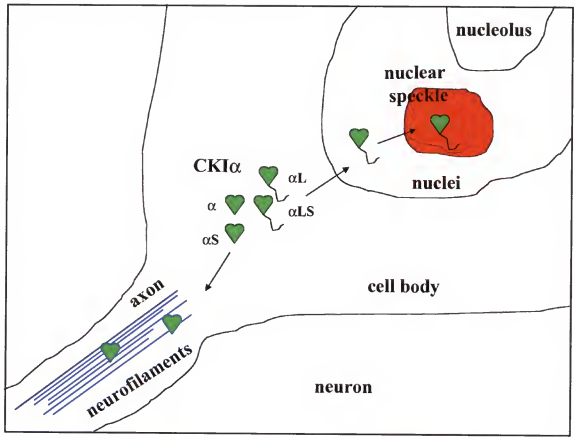


Figure 5-1. Proposed model of the differential sorting and potential functions of the four CKI alpha isoforms in neurons. CKIα and CKIαS associate with NFs and phosphorylate NF-M tail at five specific sites, whereas CKIαL and CKIαLS are targeted into the nucleus and associate with nuclear speckles, thus may play a role in the regulation of transcription and/or splicing.

compartmentalization of CKIαL and CKIαLS to nucleus, and their association with nuclear speckles that store splicing factors and RNA polymerase II. These findings not only suggest that these two specific CKI isoforms may be involved in the regulation of RNA splicing and metabolism, but also help us to understand more about how differential subcellular localization of different isoforms of a kinase specify and regulate their biological functions. Based on our findings, a model is proposed in Fig. 5-1. It should be noted that further analysis is required to confirm the nuclear localization of CKIαL and CKIαLS in neuronal cells.

Perspectives

CKI Alpha Phosphorylation and NF Functions

Previously, our lab has identified as many as 25 and probably 26 in vivo phosphorylation sites located at the carboxyl-terminal tail domain of chicken NF-M. Most of them are phosphoserine associated with a nearby proline, only the five CKI sites are not, and they are situated at the far N-terminal end of the tail, thus closer to the central rod domain. It has been demonstrated that although the entire tail domain of NF-M and NF-H is not required for NF assembly (Wong and Cleveland, 1990), the assembled NFs lacking the tails of NF-M and NF-H do not exhibit the characteristic interfilament cross-bridges (Hirokawa et al., 1984; Hisanaga and Hirokawa, 1988). Conceivably, electrostatic repulsions between NF side arms due to the presence of the many phosphates in the tail may affect the interfilament spacing and thus modulate axonal

caliber (Nixon and Sihag, 1991; Shaw et al., 1986), and many lines of evidence have suggested that it is likely to be the case (de Waegh et al., 1992; Hsieh et al., 1994).

Given that CKI sites together only occupy a small portion of total phosphorylation sites in the NF-M tail, it is likely that the phosphorylation state of those five CKI sites may not significantly change the overall charges of the tail. However, it is noteworthy that phosphorylation sites on the carboxyl-terminal tail may be functionally heterogeneous, which is suggested by the appearance of new phosphate groups late in development and differential turnover of phosphate groups during axonal transport (Dahl et al., 1986; Lee et al., 1988). So the relative ratio of the five CKI sites to the total can be varied. It is possible that, at a certain stage of neuronal development and/or under certain physiological conditions, the selective turnover of phosphates favors the CKI sites to be so predominant that they may become the major determinant of the charge property of the NF-M tail. So far, several types of phosphatase have been suggested to associate with and dephosphorylate NF proteins (Pant and Veeranna, 1995), but it remains to be determined as to which one(s) could be involved in counterbalancing the activity of the NF tail kinases. A better understanding of the coordination between the activities of NF-M tail kinases and phosphatases would help to further reveal the potential functions of the five CKI sites.

To define the functions of the CKI sites, it would be useful to generate mutant NF-M constructs with all or a subset of CKI sites mutated to alanine. Either endogenous NF-M deficient cell lines or NF-M knock-out mice may be transfected with those mutants, as well as normal NF-M, to determine if there is any abnormal NF behavior associated with any of the five CKI sites.

It has been previously documented that phosphorylation of tail domains of NF-M and NF-H can modulate the interaction between NFs and MTs (Hisanaga and Hirokawa, 1989; Hisanaga et al., 1991). Therefore, it is possible that phosphorylation of the five CKI sites might play a role in the regulation of interactions between NFs and other cytoskeletal components. To test this hypothesis, we may start with searching for potential binding-proteins that selectively bind to NFs with mutated CKI sites versus NFs with intact CKI sites, and vice versa.

CKI Alpha and mRNA Transcription / Splicing

Identification of the association of two nuclear CKI isoforms with nuclear speckles raised the questions as to how they get there and why they are there.

As detailed in chapter 4, there is an NLS motif (PVGKRKR) identified in the L insert that is likely to account for the L insert-directed nuclear targeting. On the one hand, to prove this NLS is necessary for nuclear localization, site-directed mutagenesis can be used to mutate all or a subset of the four basic residues, and see if the normal nuclear targeting can be disrupted. On the other hand, to prove this NLS is sufficient for nuclear localization, the NLS sequence can be engineered together with a cytosolic protein, and see if the chimeric protein can still be targeted to the nucleus.

Previously, a study of the *Drosophila* splicing factor Transformer (Tra) revealed that a repeating arginine/serine (RS) dipeptide sequence adjacent to a short stretch of basic amino acids was sufficient for directing it to nuclear speckles (Hedley et al., 1995). One or more copies of this motif have also been identified in a number of other splicing facors that localized to nuclear speckles. In addition, a two-step subnuclear localization mechanism was proposed, including (1) transport across the nuclear envelope, which is

mediated by the NLS; (2) association with nuclear speckle components via the RS dipeptide motif. A search of the CKI alpha sequence did not reveal any RS dipeptide sequence, suggesting that a different binding motif might cause the association of the nuclear CKI alpha isoforms with nuclear speckles. One question that can be easily addressed is whether or not the L insert contains the information for nuclear speckle targeting. In brief, the L insert can be engineered behind the GFP sequence, and the chimeric construct GFP-L is transfected into COS-7 cells to see if it co-localizes with nuclear speckles.

The current view of nuclear speckles is that not only do they serve as the storage sites for splicing factors and RNP-II, but they are also actively engaged in RNP-II mediated transcription and splicing. Since both RNP-II and several SR-rich splicing factors have been shown to be substrates for CKIα in vitro (Dahmus, 1981; Gross et al., 1999), the question would be if they are also physiological substrates for CKIαL and/or CKIαLS. The yeast two-hybrid system can be used to screen interacting partners with CKIαL and/or CKIαLS to see if RNP-II and/or any splicing factor can be identified. Meanwhile, we may transfect CKIαL and/or CKIαLS, as well as their dominant-negative forms ($K^{38}\rightarrow R^{38}$ or $D^{131}\rightarrow N^{131}$) (Peters et al., 1999; Zhu et al., 1998), to see if the CKI activity can result in the hyperphosphorylation of RNP-II and/or splicing factors. Since phosphorylation of RNP-II and splicing factors are important in regulating transcriptional and splicing activities, the establishment of RNP-II and splicing factors as physiological substrates for CKIαL and CKIαLS would support the view that these two specific CKI isoforms are involved in the regulation of transcription and/or splicing.

CKI Alpha and Signal Transduction

Phosphoinositide signaling pathway in nuclei is recently found to be closely associated with nuclear speckles (Boronenkov et al., 1998). It is known that PIPKs (phosphatidylinositol phosphate kinases) synthesize phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) by phosphorylating phosphoatidylinositol 4-phosphate (PI4P). Interestingly, multiple isoforms of PIPK were found in the nucleus and concentrated at nuclear speckles (Boronenkov et al., 1998). Meanwhile, studies from S. *pombe* revealed that phosphorylation by a yeast CKI homologue Cki can decrease the activity of phosphatidylinositol 4-phosphate 5-kinase in vitro, and more importantly, overexpression of Cki in S. *pombe* results in a reduced synthesis of $PI(4,5)P_2$ (Vancurova et al., 1999). Given that we have shown CKIαL and CKIαLS selectively associate with nuclear speckles, it is conceivable that they may phosphorylate the nuclear isoforms of PIPK, and thus down-regulate the production of $PI(4,5)P_2$ in the nucleus. Since PIP_2 produced by PIPKs can be converted to second messengers, inositol triphosphate and diacylglycerol, the functions of CKIαL and CKIαLS may possibly be extended to the regulation of nuclear PKC activity and intranuclear Ca^{2+} levels. The elucidation of the role of PIP_2 generated at nuclear speckles will provide more insights into how CKIαL and CKIαLS participate in the regulation of transcription and/or splicing.

Recently, CKI is linked to the Wnt signaling pathway in *Xenopus* (Peters et al., 1999). A *Xenopus* CKIε isoform was identified to be able to reproduce several properties of Wnt signals, including generation of complete dorsal axes, stabilization of β-catenin and induction of Wnt signal target genes. Because the *Xenopus* CKIε shares greater than 97% homology to human CKIε, it is possible that vertebrate CKIε is also essential for Wnt

signaling cascade in vertebrates. More to our interest, CKIα is also claimed to be able to induce the formation of a complete secondary axis, implying that CKIα may also play a role in Wnt pathway. There is no doubt that a further characterization of the role of CKIα in the Wnt pathway will promote a better understanding of the mechanism of Wnt signaling and more importantly of Wnt pathway-dependent developmental events and tumorigenesis.

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BIOGRAPHICAL SKETCH

In 1971, I was born in TianJin, a very large industrial city in northeast China. My father, Ru-Lian Fu and my mother, Guang-Hua Fu, both professors of physics, are engaged in research and teaching. In 1990, I graduated from one of the most prestigious high schools in the nation, NanKai Middle School, with a strong interest in studying biology. The same year, I was accepted by the Life Science Institute at NanKai University, one of the top ten colleges in China, and I received my B.S. degree in molecular biology in July 1994. In August 1994, I entered the Department of Anatomy and Cell Biology in the College of Medicine at the University of Florida for doctoral study in cell and developmental biology. My research is focused on neurofilament phosphorylation and the characterization of four casein kinase I alpha isoforms.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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